University of Alberta

The Effects of Sulphur Amino Acid Supplementation in the Early-Weaned Piglet

By

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In

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Abstract

The Effects of Sulphur Amino Acid Supplementation

in the Early- Weaned (EW) Piglet

NRC (1998) recommends that the ratio of methionine (MET) and cystine (CYS) for EW piglets is 1:1 and the concentration of total sulphur amino acids (TSAA) is 0.50 g/kg/day. However, there is little empirical data regarding the TSAA requirements for 3-5 kg piglets. Glutathione (GSH), derived from CYS, is an important biological compound that acts as an antioxidant and maintains intestinal health and cellular redox status. Objectives of this research were to examine the effects of various concentrations and ratios of MET: CYS in the EW piglet and the effect of increasing dietary cysteine on GSH synthesis. The ideal requirement for the TSAA was determined to be 75% of NRC with a 2:1 ratio MET: CYS. Inclusion of CYS above 50% of MET inclusion is detrimental for growth, intestinal development and mucosal GSH synthesis in the EW piglet.

It is not the critic who counts; Not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; Who strives valiantly; Who errs, and comes up short again and again, because there is no effort without error and shortcoming; But with who does actually strive to do the deeds; Who knows the great enthusiasms, the great devotions; Who spends himself in a worthy cause; Who at the best knows in the end the triumph of high achievement, and who at the worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who know neither victory or defeat.

Theodore Roosevelt

"When choosing between two evils I always like the one I haven't tried before"

Mae West

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka' but rather 'hmmm..... that's funny....'"

Isaac Asimov

"Science seldom renders men amiable; women, never!"

Edmone-Pierre Chanvot de Beauchene (1748-1824)

Dedication

For my parents.

From the beginning they taught me the value of knowledge for knowledge sake. They taught me that confidence in myself was a powerful tool. They taught me the value of sticking to something even when I was not good at it and that overcoming difficulties was what built the soul and nourished the spirit. They gave me the tools and the freedom to explore the world and provided me the opportunities to venture into the unknown. I know that there is no way that I can ever repay them for all that they have done for me and I can only hope that they are as proud of me, as I am in being their daughter.

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I would like to give props to my younger brother Desmond. Without whom I would have never learned to share and who, even in the darkest hours, can *always* make me laugh (I do *not* work in a piglet concentration camp) and never lets me forget that education comes from life.

Last but not least I must thank my friends. Francesca Wong, Leslie Yaremko, Craig Candler, Andrea Dichesneau, Randy Boissinant , Jason Watt and countless others who listened to my rants, laughed at my jokes and let me share in their triumphs, joys and lives. To everyone, I can never pay enough to return all that you have given me. In the end I hope that I have been as beneficial to you as you have to me. I will never forget all that I have gained from each of you and I will carry it with me always. May peace, love and joy follow you all of your days.

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1.0 Introduction

In hog production, weaning is an integral pivot point upon which the hog cycle hinges. It is after weaning that sows are rebred and piglets are raised for market. In an effort to increase the efficiency of swine production, producers in North America are utilizing a management strategy known as segregated early-weaning. This strategy, utilizing the growth potential of the young pig, removes piglets from the sow at some age less than 21 days as opposed to the more common 28 days (Alberta Agriculture, Food and Rural Development. 2001). However, as with all young mammals, piglets are designed to consume milk as their primary nutrient source. At weaning, the abrupt change in diet results in a growth lag (Robert et al., 1999).

In an effort to ameliorate the growth lag, nutritionists design rations that are highly digestible, palatable and that meet the nutritional needs of these very young animals. Many of the feedstuffs used by nutritionists, while palatable, contain low concentrations of the sulphur amino acids, methionine and cysteine. Also, many of these feedstuffs, such as oat groats and skim milk powder, contain more cysteine than methionine (NRC, 1998); the effects of excess cystine on the gastrointestinal development of segregated early-weaned piglets are unknown.

Derived from cysteine, glutathione is a ubiquitous tri-peptide involved in a number of important biological processes: maintaining cellular redox status, as anti-oxidant and an amino acid transporter (Droge and Brietkreutz, 1999). Glutathione is a known mucolytic agent that acts to increase the fluidity of the mucus lining the lungs,

increasing the ease of expectoration in Cystic Fibrosis patients (Rubin, 2002). Increased dietary cysteine intakes may increase the production of gastrointestinal glutathione and thereby affect the viscosity and integrity of the intestinal mucosal lining. This in turn may expose the small intestine to bacterial translocation and reduce the adaptability of the early-weaned piglet.

Because early-weaned piglets are less gastro-intestinally developed than their later weaned counterparts and that nutritionists utilize feedstuffs that often contain excess cystine, investigation into the effects of total sulphur amino acid inclusion and the effects of excess cystine on growth and gastro-intestinal development in the early-weaned piglet is essential.

2.0 Literature Review

2.1 Weaning

Weaning of piglets in the wild is a gradual process that naturally occurs over a 16-18 week period. Due to increased demand by the piglet and a dilution of the protein density (Pajor et al, 1991), the sows' milk becomes less able to support piglet growth. In order to meet their nutrient requirements, piglets engage in rooting and foraging behaviours to secure alternatives to their milk diet, gradually adapting their digestive tracts to the digestion of solid feed (Alberta Agriculture, Food and Rural Development, 2001).

Traditionally in swine production, commercial producers wean piglets between 3-6 weeks of age. Often, the piglets are moved to a location on farm, mixed with other litters and are abruptly adapted to eating a solid, grain-based diet. At weaning, piglets are vulnerable to disease and experience decreased adaptive ability due to exposure to a wide variety of stressors (Maxwell and Sohn, 1999). These stressors, namely changes in diet and environment, result in a post-weaning growth lag that is characterized by a reduced ability to grow optimally due to reduced feed intake and reduced digestive capability in the piglet (Leibbrandt et al., 1975, Okai et al., 1978). One goal of nutritional weaning research is to examine the effects of weaning on the post-weaning growth lag and to determine if nutritional intervention, such as amino acid supplementation, can ameliorate the inhibition of piglet growth during this time.

2.1.1 Segregated Early Weaning (SEW)

As opposed to traditional weaning, early weaning (EW) is defined as the removal from the sow at less than 21 days old and movement of piglets to a location on- or off- farm that has been rigorously cleaned and disinfected prior to piglet entry (Pyburn and Schwartz, 1995).

While it is only in the last 5-10 years that EW has become popular with producers in Alberta, the concept of EW is not novel. In their book 'Nutrition of the Young Pig', Lucas and Lodge (1961) discuss the dietary needs of the early-weaned pig and the many issues associated with this management strategy. For many years the idea of EW was largely ignored due to poor rates of gain observed in piglets weaned prior to 3 weeks of age compared to their traditionally weaned counterparts (Liebbrandt et al., 1975). However a paper published by Alexander et al. in 1980, stimulated renewed interest in this strategy. Alexander et al. (1980) outlined that, if antibiotics were supplied prophylactically disease transmission such as transmissible gastroenteritis, could be avoided and EW piglet growth could be maximized while mortality was minimized. Vertical disease transmission from the sow could be avoided through the removal of piglets when the immunity derived from the sows' colostrum was high and horizontal transmission, the transmission of disease from older to younger piglets, could be avoided through the segregation of the piglets on the basis of age (Robert et al., 1999). With the introduction of segregation by Harris (1988) that is, the raising of piglets in isolation, came a refinement of the early weaning management strategy that resulted in the development of segregated early weaning (SEW).

Harris (1988) altered the SEW concept to make it more practical than Alexander et al. (1980) antibiotic-intensive procedure. Harris noted that medication levels could be reduced for piglets weaned as early as 10 days, if they were raised under clean conditions. Raising piglets in environments that met strict cleanliness requirements reduced the bacterial load to which they were exposed. Cleanliness allows the piglet to channel nutrients towards growth rather than towards an immune response, thus reducing the negative impacts of reduced immunity at weaning and the inclusion of expensive antibiotics (Fangman and Tubbs, 1997).

2.1.2 Piglet Weight Gain

Early studies that examined the weaning of pigs demonstrated that piglets weaned early were not comparable to their nursing counterparts in regards to growth. Furthermore, an increase of weaning age allowed for a 'smoother' adaptation by the pigs to their new environment resulting in greater weight gains, fewer days to market and lower mortality (Leibbrandt et al., 1975). Later work, like that of Alexander et al., (1980) and Harris (1988), reported increased growth responses of early-weaned pigs versus their traditionally weaned counterparts when raised following strict guidelines of medicinal intervention or cleanliness (Robert et al., 1999). Dritz et al., (1994) weaned piglets at 9- and 19-days and fed 3 diets of variable ingredient complexity and nutrient levels. These researchers found no differences between the growth rates or feed conversion rates of either group raised under similar management. These researchers concluded that by maintaining a clean environment and formulating a diet that encourages consumption and therefore growth, piglets

weaned at 9-days of age, could grow identically through to market weight as animals weaned at a later date.

During a period of reduced feed intake, such as that found at weaning due to changes in diet and environment, animals lose weight. When piglets return to eating they have a greater efficiency of energy use, a higher growth rate and they gain less fat for each unit of gain (Abdalla et al., 1988). This is known as compensatory gain and is characterized by a rapid and efficient growth after a period of feed restriction (Sainz et al., 1995). While this period of efficient nutrient utilization makes early-weaning advantageous the preceding growth lag often results in high mortality (Robert et al., 1999), therefore avoidance or amelioration of the growth lag is desirable.

2.1.3 Piglet Immunity

At birth, piglets possess little or no active immunity (Miller et al., 1961). Antibodies are provided via colostrum and are absorbed in the gastrointestinal tract of the piglet over the first 24-36 hours of life (Brown et al., 1960). The maternally-derived antibodies are detectable in the blood of the piglets until 6 weeks but show a marked decrease after the third week. After 3 weeks of age, the piglets' own immune system begins actively producing antibodies against pathogens to which it has been exposed (Miller et al., 1961). However, at 3 weeks of age there is a period of time when there is a depression in passive immunity and active immunity is still low, known as an immunity lag. Traditionally, piglets are weaned during this immunity lag period and are thus more vulnerable to pathogenic attack. The removal of piglets prior to the immunity lag allows piglets to adapt to a new environment and reduce the exposure

risk to common endemic pathogens present in the breeding herd (Fangman and Tubbs, 1997). Piglets can then adapt to a new social structure, environment, diet, and begin gaining weight during this period of high passive immunity as opposed to piglets who are weaned later during the immunity lag and must suffer the added stress of immature immune status.

Piglets raised under SEW management are more efficient at gaining weight than their traditionally-weaned counterparts. This has been reportedly due to less energy being diverted to immune activity (Tang et al., 1999). Because SEW takes advantage of passive immunity from the sow, housing in clean conditions and not mixing older and younger pigs, new entries into a SEW unit are not confronted with a pathogen overload (Fangman and Tubbs, 1997). When challenged, the production and release of cytokines from activated macrophages negatively affects growth (Webel et al., 1997). Cytokine production repartitions dietary nutrients away from growth towards immune function by recruiting immune modulators like glucocorticoids, prostaglandins and catecholamines, that act to decrease protein accretion and increase protein degradation (Spurlock, 1997). There is also a reduction in feed intake in the presence of cytokines (Johnson et al., 1992). This reduction in feed intake coupled with increased degradative mechanisms, requires the pig to draw on body stores, further decreasing the muscle mass of the animal and deepening the post-weaning growth lag. The greater the weight loss during the growth lag the longer the return to the piglets' physiological set weight and a longer time to reach market weight results.

Developing dietary interventions that prevent or alleviate this common situation are necessary to improve the success of early weaning.

2.1.4 Stress

Removal from the sow, mixing with non-littermates and introduction to a new environment are all stressors that negatively impact SEW piglet growth (Dybkjaer, 1992). Stress is defined as: the requirement of an animal to make abnormal or extreme adjustments to its physiology or behaviour to survive negative environmental factors (Fraser et al., 1975). As outlined earlier, natural weaning is a gradual process over which time the sow becomes less capable of supplying 100% of the piglets dietary requirement and the piglet is forced to find alternative nutrient sources to meet their dietary need (Worobec et al., 1997). The earlier and more abruptly piglets are weaned the more they exhibit negative stereotypic behaviours such as belly nosing, navel sucking, head shaking or chewing (Dybkjaer, 1992). Other such coping mechanisms are aggressive behaviours such as fighting. Pigs live in hierarchies that determine group behaviours (Hicks et al., 1998). SEW piglets appear to be much more aggressive in stereotypic and fighting behaviours that establish these hierarchies than traditionally weaned piglets (Worobec et al., 1997). Worobec et al., (1997) studied the behaviour of pigs weaned at 7, 14 and 28 days of age and demonstrated that piglets weaned at 7 days were lighter at 6 weeks of age and exhibited more escape and aggression behaviours than did 14- or 28-day weaned pigs. These researchers used this evidence to suggest that SEW reduces both performance and welfare of SEW piglets. However, the authors ignored the fact that the heaviest pigs at 6 weeks were the 14-day (early-weaned) pigs. It was more likely a function of the

nutritional needs of the 7-day weaned piglets not being met by the same diet that was fed to older piglets, than the effects of stress due to early-weaning. The effects of stress on the gastrointestinal development of the early-weaned piglet must be considered when designing management strategies and when formulating diets.

2.2 Gastrointestinal Development

2.2.1 Physical Development

During gestation, the fetal small intestine develops from a smooth surface to having villi and microvilli forming an absorptive layer past which nutrients flow and are extracted (Neu and Koldovsky, 1996). Enterocytes, the absorptive unit of the digestive tract, originate at the base in the crypts of Leiberkühn. These cells mature as they migrate the length of the villus but are short-lived as the replacement of cells, from crypt to villus tip, takes 24-96 hours (Goke and Podolsky, 1999). During the suckling period, villus height decreases while microvilli number, crypt number and depth increase (Weaver and Carrick, 1989). The ability to absorb macronutrients and immune factors from colostrum ends after 24-36 hours of life (Attaix and Meslin, 1991) when the junctions between the enterocytes close.

At birth, neonatal piglets receive colostrum by suckling the sow. Colostrum differs from mature milk in that colostrum has a higher concentration of protein, fat and lactose (Widdowson, 1985). The proteins present in colostrum are mainly immunoglobulins and growth factors that are vital for the piglets' survival (Weaver, 1997). These growth factors stimulate cell growth and the expression of

differentiation of cells in the gut (Odle et al., 1996). Widdowson et al. (1972) described the growth of the small intestine during the first 24 hours of life in piglets fed with water or colostrum. The water-fed piglets had lower duodenal, jejunal and ileal weight and weight per length of gut than the colostrum-fed piglets. These differences may be a function of increased protein synthesis and hypertrophy of the small intestine due to the absorption of immunoglobulins and growth factors from colostrum (Burrin et al., 1992). The development of the gut due to colostrum is necessary as gut architecture is not only required for the absorption of nutrients but also because the gut acts as a barrier to disease. If piglets receive little or no colostrum there is a delay in the closure of the junctions between the enterocytes and pathogenic bacteria translocation can occur (Veereman-Wauters, 1996).

Regardless of whether piglets are traditionally or early weaned, when removed from the sow there is an abrupt change in diet. The piglet becomes anorexic after weaning; this results in a loss of weight and a loss of intestinal and immune function (Robert et al., 1999). This anorexia can be explained by diet-dependent and -independent means (McCracken et al., 1999).

The change in environment, mixing with non-littermates and mode of feeding all act as diet-independent influences on GI digestion and absorption. Piglets move from hourly, liquid feedings while on the sow to ad libitum, solid feeding (Worobec et al., 1997). Piglets must first be able to find the feeder and determine what purpose it serves prior to being able to eat. This delay in eating alters the small intestine villus

structure, by decreasing the length and down regulating enzyme activity (Hampson and Kidder, 1986). The alteration of the villus structure decreases the absorptive surface area while the decreased enzyme activity decreases the availability of circulating nutrients from the diet (Hampson, 1986).

2.2.2 Development of Enzymes

The ingestion of colostrum by piglets also serves to stimulate enzyme activity, specifically lactase, in the small intestine (Burrin et al., 1994). There is an alteration in the enzyme secretions over time that aid in digestion as the diet of the piglet changes. From birth until approximately 10 days postnatally, there is a sharp increase in lactase activity. After day 10 however, there is a sharp decrease in lactase activity. Conversely, maltase and sucrase activities are low until approximately 10-15 days postnatally, after which activities increase. This changing enzyme pattern reflects the piglet diet pattern. While milk makes up 100% of the piglets diet early life, milk quickly becomes less able to meet the nutrient and energy requirements and the piglet is forced to seek alternative sources (Kitts et al., 1955). These alternative nutrient sources, such as creep feed, act to capitalize on the growth potential of the piglet by not limiting its access to nutrients or energy (Worobec et al., 1997).

Enzymatically, piglets that are early-weaned have a reduced level of lactase and increased level of sucrase and maltase activity compared to their suckled counterparts of the same age. This alteration in the pattern of enzyme activity is due to changes in diet as well as age. Piglets that are allowed to suckle have similar changes in GI

enzyme profiles as their EW counterparts; however, this enzyme profile change is induced by EW, either via change in diet or due to a stress response (Thacker, 1999) and therefore occurs earlier. In a study by Kelly et al. (1991) piglets were separated into 2 groups, early weaned and sow-fed. The early-weaned piglets were weaned at 14 days and killed after 3, 5 and 7 days on trial while the sow-fed groups were slaughtered at 14 and 22 days. The early-weaned group were fed using gastric intubation that allowed piglets to be fed at 3 h intervals thereby allowing a direct comparison with their sow-fed littermates. As both the sow-fed and SEW piglets aged lactase activity fell and sucrase and maltase activities increased. Although the pattern of change was similar between the groups, the early-weaned piglets had precocial alteration of the enzyme profile in contrast to their sow-fed counterparts. While precocially induced, the diets of SEW piglets must be nutritionally graded to reflect the pattern of change. Initial SEW diets must be high in lactose to reflect the initially high levels of lactase enzyme present. However, over time the level of lactose in the diet is decreased to reflect the maturation of the digestive enzyme profile of the piglet.

2.2.3 Function and Immunity of the GIT

Beyond acting as a digestive and absorptive organ the gut also acts as part of the immune system (Insoft et al., 1996). While immature at birth, the gut develops during the suckling period to prevent pathogens or antigenic material from harming the small intestine or entering the systemic circulation (Tang et al., 1999). Protection is based on immunological and non-immunological barriers (Insoft et al., 1996). The primary action in the prevention of insult to the GIT is in the prevention of adherence

by pathogens to the gut wall (Cummins and Thompson, 1997). These 'anti-adhesive' factors, such as IgA, are secreted along the small intestine from intestinal epithelial cells, occupy receptor-binding sites and prevent adhesion (Insoft et al., 1996). In the neonate the gut is not able to produce large amounts of IgA, but the factor is available from the mothers' milk that acts as the primary source for the piglet (Cummins and Thompson, 1997).

Diet-dependent influence on the post-weaning growth lag may be due to the sensitisation of the gut to plant proteins (McCracken et al., 1999). When exposed to nursery diets containing soybean proteins, piglets that subsequently receive starter diets containing soybean proteins have an immune response due to sensitivity developed to these proteins during the nursery period (Hampson and Kidder, 1986). Piglets that do not receive creep feed or sow feed during the suckling period do not experience the inflammatory immune response. Pigs that receive nursery diets with milk replacer rather than soy-protein weigh more and have longer small intestines and greater intestinal surface area for absorption (Zijlstra et al., 1996). Also, these piglets do not experience the inflammatory response, because they have had no previous contact with the plant proteins. Therefore, when creep feeding piglets it is important that the diet composition be carefully determined to avoid compounding any anorexic behaviour during the weaning phase.

2.3 Amino Acids

Amino acids are divided into two groups, indispensable and dispensable. Indispensable amino acids are those that animals cannot synthesize endogenously and

therefore require a dietary source. Dispensable amino acids are those amino acids that can be synthesised endogenously or derived from dietary precursors.

For pork producers, the gain of muscle mass by the pigs is of primary importance. In order to ensure gain, diets are carefully formulated to meet the nutritional requirements of the animal. Researchers have long studied the requirements for indispensable amino acids by measuring weight gain, nitrogen balance and feed efficiency. With insufficient intake of indispensable amino acids, such as that observed during the weaning period when feed intake is decreased or ceases, animals do not to grow. In an effort to maintain biologically required circulating concentrations of indispensable amino acids, animals will sacrifice muscle mass to liberate amino acids to perform other vital biological functions apart from skeletal muscle protein synthesis. Therefore research into nutrients, such as amino acids like methionine or cystine, are essential to ensure productivity on farm.

2.3.1 Methionine

Methionine (MET) is an indispensable sulphur-containing amino acid (Lehningher et al., 1993). MET receives the classification of indispensable because animals cannot synthesize MET de novo as they can other amino acids such as glycine or arginine (Becker et al., 1954). Dietary MET is the primary source of MET while protein degradation and MET remethylation provides recycled MET (Livesay, 1984). MET is involved in a number of biologically important processes of which, protein synthesis, methyl donation and cysteine/cystine (CYS/CYS_2) synthesis are a few (Stipanuk, 1986).

2.3.2 Methionine Requirements

In 1954, Becker et al., recommended that the MET level in a diet of growing pigs be 0.25% in the presence of 0.17% CYS_2 for a diet that is 12.6% protein. Later, Kroening et al., (1965) determined a level of 0.52% MET + CYS_2 in a diet of 12% protein for pigs aged 2.7 weeks. In 1984, Liebholz recommended methionine be included at 3.0 g/kg DM (0.26% of a 26% crude protein diet) for pigs between 7-28 days of age for maximum growth performance and nitrogen retention. This recommendation is lower than that suggested by Kroening et al., (1965) and the American Research Council (ARC) (1979). The differences in the estimates of the requirements are most likely due to differences in diet composition, variable protein concentrations and ingredient, and the age of pigs studied. More recently, Chung and Baker (1992a) determined a total sulphur amino acid requirement (TSAA) of 0.58% in a diet of 20% crude protein for pigs from 5-20 kg BW. Chung and Baker (1992a) reported that CYS_2 could spare up to 50% of MET, and deduced that 0.29% of the TSAA should come from MET. In 1998, the National Research Council (NRC) recommended that MET be available at 0.36% of the diet for pigs 3-5 kg, in diets that are 26% crude protein. Most recently Shoveller et al. (2003a) reported that piglets weighing 3 kg require ~0.53% of a diet in an 18% crude protein diet based on purified ingredients.

While MET is indispensable for the pig, care must be taken with MET supplementation. MET excesses are reached at intakes of approximately 2-2.5x the TSAA requirement and methionine is considered the most toxic of all the amino acids

(Harper et al., 1970). The negative effect of excess MET intake is due to alterations in the metabolism of the methyl group. Excess MET results in excess methyl groups that cannot be converted to CO_2 for safe removal from the body (Benevenga, 1974). Excess intakes of MET results in decreased feed intake in chicks (Katz and Baker, 1975) and weight gain in rats (Regina et al., 1992). Prolonged intakes of excess MET can lead to renal, hepatic (Regina et al., 1992) and splenic (Benevenga et al., 1976) damage and failure.

While NRC (1998) is the benchmark against which commercial diets are formulated, there are difficulties in isolating the exact requirement of MET in the diet that is of the most benefit. NRC gathers all of available research and attempts to equate experiments to each other to determine an average requirement as percentage of the diet (NRC, 1998). The difficulty associated with this practice is that in attempts to be novel, researchers have created numerous diet types or used genetically diverse pigs under various rearing conditions with inconsistent health statuses. These inconsistent research variables conspire to make determining an adequate requirement for MET in pig diets difficult. Another difficulty with the NRC requirement is that the main research models in determining requirement are that of nitrogen balance or growth rate. The nitrogen balance methodology has numerous inherent problems that either act to overestimate requirement (van Barneveld et al., 1995). Also, for pigs weighing 3-5 kg there is little experimentation done and the stated values are extrapolations of existing requirements for heavier, older animals.

2.3.3 Biological functioning of Methionine

While MET plays roles in a multitude of biological functions, one of the most important is protein synthesis. The MET content of a young pig was found to be 2.3 g /100 g of crude protein (Liebholz, 1984). Battersham et al., (1993) reported that the concentration of MET in the carcass depends on the concentration of MET in the diet. That is, low dietary MET availability results in lower carcass concentrations of MET. It appears that, to date, accretion studies have been based on the creation of a non-limiting diet from a diet that is limiting in some essential nutrient. The data from these studies, while showing that certain nutrient factors increase performance when supplied adequately, does not provide a complete understanding of how nutrients with complex metabolisms, such as MET, act to increase performance.

Wu, (1998) published a review paper documenting gastrointestinal metabolism of amino acids. This paper showed a paradigm shift from Rose in the '50's where the gut was not recognised as an amino acid utilizing or catabolising organ rather, but as a tube along which digesta passed and across which nutrients were absorbed. Once in the enterocytes approximately 50% of the available dietary MET disappears. If measured the portal blood concentration, only 48% of the dietary Met was found (Stoll et al., 1998). Stoll et al., (1998) stated that the remaining 52% of the un-transported MET fulfils some 'metabolic role' in pig intestinal mucosal cells. More recently, compartmentalization modelling has indicated that the gut may sequester amino acids for release during the postprandial state (Fouillet et al., 2000).

Tracing the pathways outlined in Figure 2.1 it is apparent that MET metabolism is complex. The diagram also illustrates the recycling of MET from 3 sources: 1) protein degradation, 2) homocysteine and 3) polyamines. The number of recycling pathways for MET acts to underscore its importance, MET exclusion from or deficiency in diets, stunts growth and development (Chung and Baker, 1992a) indicating a fundamental need, while the number of recycling pathways, not just the fact that it can be recycled, indicates that MET is for some reason of major importance beyond simple protein accretion.

Methionine, via S-Adenosylmethionine (SAM) acts as the primary methyl donor in the body (Chiang et al., 1996). This donation is accomplished via the transfer of the S-methyl group to possible acceptors via transmethylation (Lu, 2000). Once SAM donates its methyl group it is converted to S-Adenosylhomocysteine (SAH) (Finkelstein, 1990). SAH inhibits transmethylation reactions and because of this its removal via homocysteine (Hcy) is vital. Hcy can then be transulferated to cysteine or remethylated to methionine via methionine synthase utilizing folate and vitamin B_{12} or via betaine-homocysteine methyltransferase utilizing betaine (Finkelstein, 1998b). An important role of methyl donation, and of interest to gastro-enterologists, is that of endogenous polyamine synthesis. Polyamines aid in the regulation of cell homeostasis, and play an important part in cell proliferation, differentiation and hyperplasia (Sousadias and Smith, 1995). Animals are first exposed to polyamines during suckling, where exposure aides developmental changes as the neonate moves from immaturity to maturity (Grimble and Grimble, 1998). In studies that

from immaturity to maturity (Grimble and Grimble, 1998). In studies that supplemented dietary polyamines, incidences of diarrhea in children from poverty stricken areas was lowered by 16%. This decrease was believed to be due to polyamines inhibiting bacterial translocation and changes to the intestinal mucosa that can cause diarrhea (Grimble, 1996). Although it appears that dietary polyamines from milk are the more prevalent type (Bardocz et al., 1995) for inducing precocious development of the gut (Grimble and Grimble, 1998) it appears that supplementation of polyamines into the diets of early-weaned piglets does not enhance gastrointestinal maturation (Ewtushik et al., 2000). The role of MET in the production of endogenous polyamine synthesis must be considered when formulating a diet for the early-weaned pig. Inducing gut development while providing intestinal mucosa protection is of great interest due to the relative immaturity and susceptibility of the early-weaned pig.

2.3.4 Cyst(e)ine

Cysteine is a sulphur containing amino acid that exists as either a single cysteine or the dimer (2 cysteines joined via a sulphide bond), also known as cystine (CYS_2) (Lehningher et al., 1993). In human nutrition, CYS is determined to be conditionally indispensable, that is, the neonate is incapable of adequate de novo synthesis but the adult can produce enough CYS to satisfy its biological requirements (Finkelstein et al., 1986). CYS is considered to be dispensable only when enough MET is available in the diet (Chung and Baker, 1992b). Neonatal indispensability is due to the low activity of the enzyme cystathionase, that is present in the liver and kidneys and is responsible for the conversion of Hcy to CYS (Zlotkin et al., 1981). It is presently

unclear if early-weaned piglets are capable of producing sufficient CYS to meet their requirement or like the human neonate are bound by limited enzyme activity. However, research in our laboratory indicates that there was no difference in phenylalanine oxidation when methionine was supplied at requirement and CYS was supplemented to meet the TSAA requirement than when MET alone met the TSAA requirement (Shoveller et al., 2003b). This indicates that CYS is not indispensable in the EW piglet. However, it remains unclear if satisfying the protein synthetic requirement also satisfies all of cystines other synthetic requirements.

CYS is involved in protein synthesis, the synthesis of the amino acid taurine, and the antioxidant glutathione (GSH) (Bella et al., 1999). CYS can also act to spare MET that is terminally shunted to the non-recyclable transsulphuration pathway for the synthesis of CYS. NRC (1998) recommends CYS_2 replace MET at a 50:50 ratio but actual experimental values range from 40% to 70% for growing pigs (Chung and Baker, 1992b). The sparing ability of CYS results from a decrease in hepatic cystathionine β-synthase, the enzyme responsible for the first step of the transsulphuration pathway that converts Hcy to CYS (Finkelstein et al., 1988). When insufficient CYS is available, homocysteine is converted to CYS rather than being remethylated to MET.

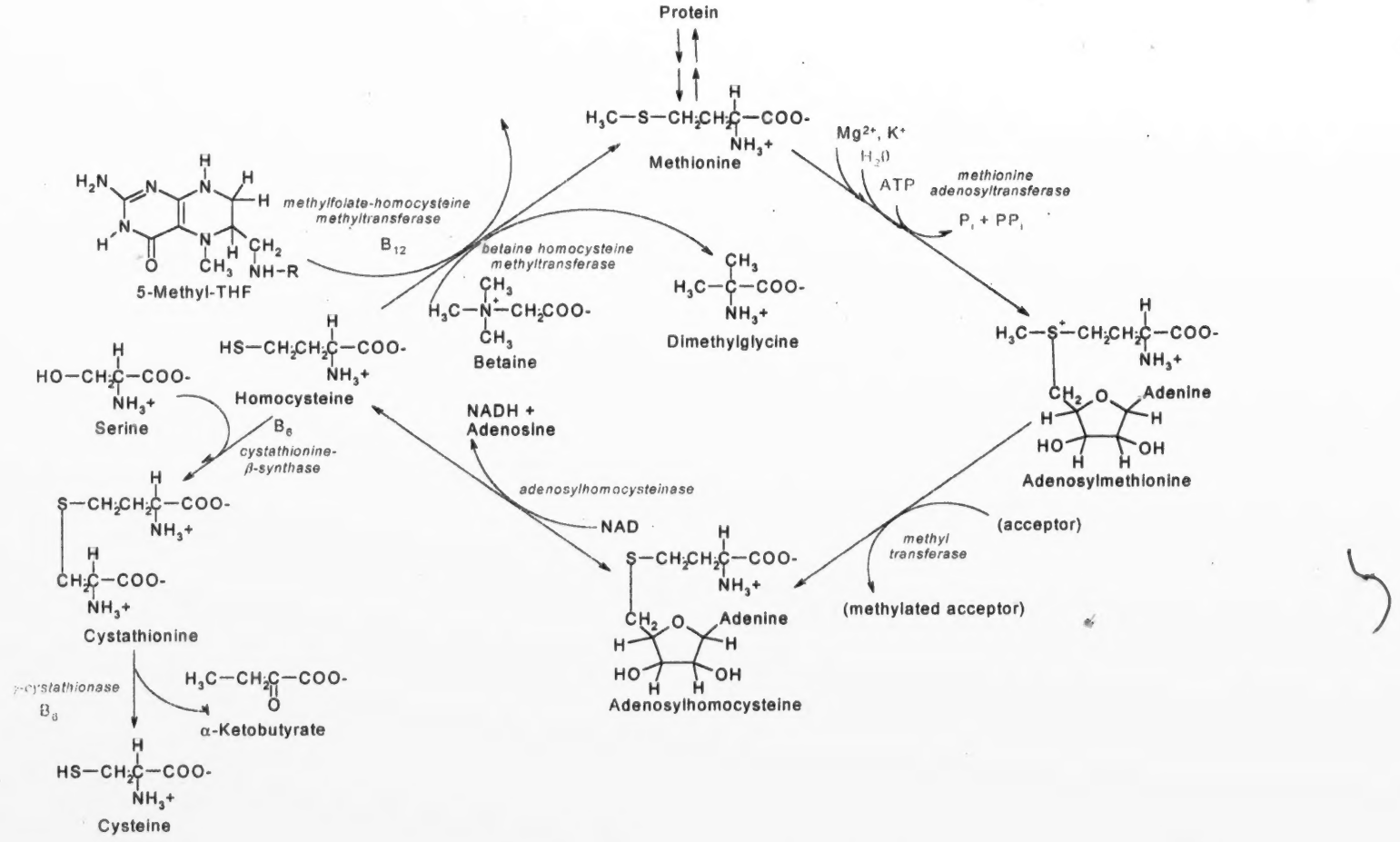
CYS is either supplied through the diet, via de novo synthesis from MET or through protein degradation (Chung and Baker, 1992b). According to NRC (1998), there is no requirement for CYS, rather it is coupled with MET and assumed to spare half of the TSAA requirement. Becker et al., (1955) reported that CYS_2 could supply 40%

Baker et al., re-evaluated CYS_2 ability to spare MET. The researchers reported that CYS_2 could meet at least 56% of the total sulphur amino acid requirement for the young pig. Chung and Baker, (1992b) formulated diets containing various concentrations of MET and CYS_2 in order to determine the maximum proportion of the TSAA requirement that could be met by CYS_2 for the young pig. When MET supplied 40% of the TSAA requirement, pigs experienced daily gains of 461 g with a gain to feed ratio (g/kg) of 544. When MET was 60% of TSAA requirement, daily gain was at 530 g with a gain to feed of 569 g/kg. The greatest gains were observed when MET and CYS_2 were present in equal percentages (50:50). Daily gain was 531 g while gain to feed was 578 g/kg.

There is little literature in regards to the toxicity of CYS_2. In the first half of the 20^{th} century, a small number of studies investigated the effects of excess CYS_2 in rat diets were reviewed by Harper et al. (1970). Decreased feed intake and gain were common observations and there was no comment of the effects of excess dietary CYS_2 intake on the gastrointestinal tract. To the best of the author's knowledge there are no recent papers (since 1955) on CYS_2 toxicity in any species.

While MET-sparing capacity of CYS is important so too are the products of CYS. Glutathione (GSH), synthesized from glutamic acid, cysteine and glycine, is one such product. The antioxidant role of GSH may be of considerable importance for the early-weaned pig. Oxygen-derived free radicals can cause tissue damage and the

Figure 2.1. Metabolic Pathway of Methionine (adapted from Finkelstein. 1990).



lining of the intestines is not exempt from this threat. Mucosal cells have GSH present and during increased free radical or superoxide pervasiveness the levels of GSH are reported are depleted (Benard and Balasabramanian, 1993). When weaned, the piglet experiences stressors that may result in increased free radical attack and evidence indicates that GSH may provide protection to the gut epithelium (Martensson et al, 1990). Further discussion of GSH occurs in a subsequent section of this chapter.

Taurine (TAU) is a β- amino acid that is not incorporated into protein. TAU is synthesized from CYS via a number of enzymatic steps to produce hypotaurine and finally taurine. TAU is utilized for the formation of bile acids. In the liver, TAU is conjugated with cholic acid to form taurocholic acid. Taurocholic acid is secreted into the bile and is used as an emulsifying agent of lipids in the intestines (Hadorn et al., 1974). TAU is also a neurotransmitter and total parenteral diets deficient in TAU for preterm infants are believed to lead to slowed developmental growth and reduced brain and retina development (Stapleton et al., 1997). Other functions of TAU include: antioxidant, membrane stabilizer and regulator of Ca^{2+} homeostasis (Stapleton et al., 1998).

2.4 Glutathione

2.4.1 Introduction

Glutathione (GSH) is a low molecular weight tri-peptide that is synthesized by all cells in the body (Anderson, 1998). GSH ubiquitous nature reflects the many

functions that GSH fulfils within the cell (Sen, 2000) where it may act as an antioxidant and an amino acid transporter. GSH is involved in the synthesis of protein and nucleic acids and in the maintenance of redox status (Valencia et al., 2001). GSH is also present in cells as glutathione disulfide (GSSG). This oxidized form of GSH is generally present at 10 to 100 times lower concentrations than that of reduced GSH (Griffith, 1999).

2.4.2 Synthesis

GSH is synthesized from 3 amino acids; glutamate, cysteine and glycine, and utilizes 2 enzymes, γ- glutamylcysteine synthetase and glutathione synthase. The following reactions (1 and 2) are the stepwise formation of GSH:

1) L-Glu + L-Cys + γ-glutamylcysteine synthetase + ATP ⟶ L- γ -Glu-Cys+ADP+P*i*

2) L- γ -Glu-Cys + L-Gly + Glutathione Synthetase + ATP ⟶ GSH + ADP + P*i*

(Dickinson and Forman, 2002)

In reaction (1) cysteine is the limiting substrate as it is dependent upon cysteine availability. Both reactions occur in the cytosol of the cell. Concentrations within the cytosol range from 1-10 mM depending upon cell type, species or health status of the animal (Pastore et al., 2003). GSH is exported from the cell but due to its size and molecular arrangement is not transported into cells as GSH. On the outside of the cell membrane is the enzyme γ-glutamyl transpeptidase that cleaves the unusual γ-glutamyl bond that exists between the glutamate and cysteine in the dipeptide glutamylcysteine (Anderson, 1998). Broken down, the constituent amino acids are

transported across the cell where the amino acids are utilized either for GSH biosynthesis or for some other function depending upon the needs of the cell (Sen, 2000). Because both glutamate and cysteine may not be dedicated to the biosynthesis of GSH after transport, GSH is considered a source of amino acids (Meister, 1983) (Figure 2.2). All cells synthesize GSH, but the liver is considered the primary organ of synthesis (Sies, 1999). The liver has the highest concentration of GSH, because, unlike other cell types hepatocytes can synthesize cysteine from methionine through the transsulfuration pathway and provide cysteine for GSH synthesis endogenously. Also, unlike other cells the liver exports most of the GSH it synthesizes, either into the plasma or bile. This last point makes the liver different from other cell types as GSH is usually exported from cells to restore the 2GSH: GSSG relationship thereby maintaining redox status, as opposed to the liver that synthesizes GSH almost specifically for export (Lu, 1998). GSH concentrations in the plasma range from 1 to 9 μM and are representative of hepatic synthesis.

In the mucosa of the intestine the GSH concentration is 50-60% that of the liver (Siegers et al., 1989) however, the intestine only synthesizes 40% of this amount (Loguercio and Pierro, 1999). The difference is the result of biliary GSH excreted into the duodenum during feeding (Dahm and Jones, 2000). The importance of GSH in the intestine is discussed later in this section.

2.4.3 GSH as an Antioxidant

All cells exist in an oxidizing environment. A consequence of this environment is the creation of a number of deleterious compounds: superoxides, free radicals and hydrogen peroxide. Although GSH does not react directly with hydroperoxides, it is used as a substrate for glutathione peroxidase (GSHPx), the predominant mechanism for reduction of H_2O_2 and lipid hydroperoxides.

Another antioxidant role for GSH is that of regenerating vitamins C and E from their oxidized states. Cytosolic GSH acts as a cofactor for membrane bound vitamin E free radical reductase that regenerates vitamin E from a tocopherol radical. Vitamin C can also act to spare GSH consumption by reducing the GSH dependent reduction of dehydroascobate to ascorbate and by acting as an alternative reducing agent. Dietary supplementation with vitamin C significantly increased the concentration of GSH in the plasma of rats (Sen, 1997).

Damage from oxidants can result in increased cellular apoptosis in the gut. This can lead to cytokine production, inflammatory responses and result in a 'leaky gut' that allows bacterial translocation to occur across the lumen (Miller et al., 2002). In piglets that are EW, the stress of weaning itself can cause these events to occur. Dietary intervention may be able to decrease the damage done to the EW piglet gut and maintain a healthy functioning gut during weaning.

2.4.4 Redox and GSH

From the cytosol, GSH is either transported within the cell or exported from the cell into tissue to maintain cellular redox status (Anderson, 1998). Redox status is important as all biological functions are derived from the energy supplied by the movement of electrons. The movement down the electron gradient results in an overall reducing environment in the cell (Schafer and Buettner, 2001). The reducing environment results in an anabolic environment but also results in the formation of superoxides ($O^{\cdot}$) that results in oxidation (catabolism). In this catabolic state, GSH acts to bind the superoxides and return the cell to a reducing state (Anderson, 1998). The 2GSH: GSSG relationship is considered the primary cellular buffer and the movement between the redox pair and the export of either GSSG or GSH from the cell maintains redox status (Filomeni et al., 2002). Maintaining concentrations of intracellular GSH is a function of use and synthesis. Depletion of GSH occurs by conjugation reactions or by GSSG formation through increased H_2O_2 production and activity. Response of a cell to a stress often involves changes in thiol content. GSH is first consumed in reactions that protect the cell by removing the deleterious compound. Then it is replaced either through enzymatic reduction of a disulfide or by de novo synthesis (Schafer and Buettner, 2001), returning the ratio of 2GSH/GSSG to optimal.

2.4.5 Gastrointestinal GSH

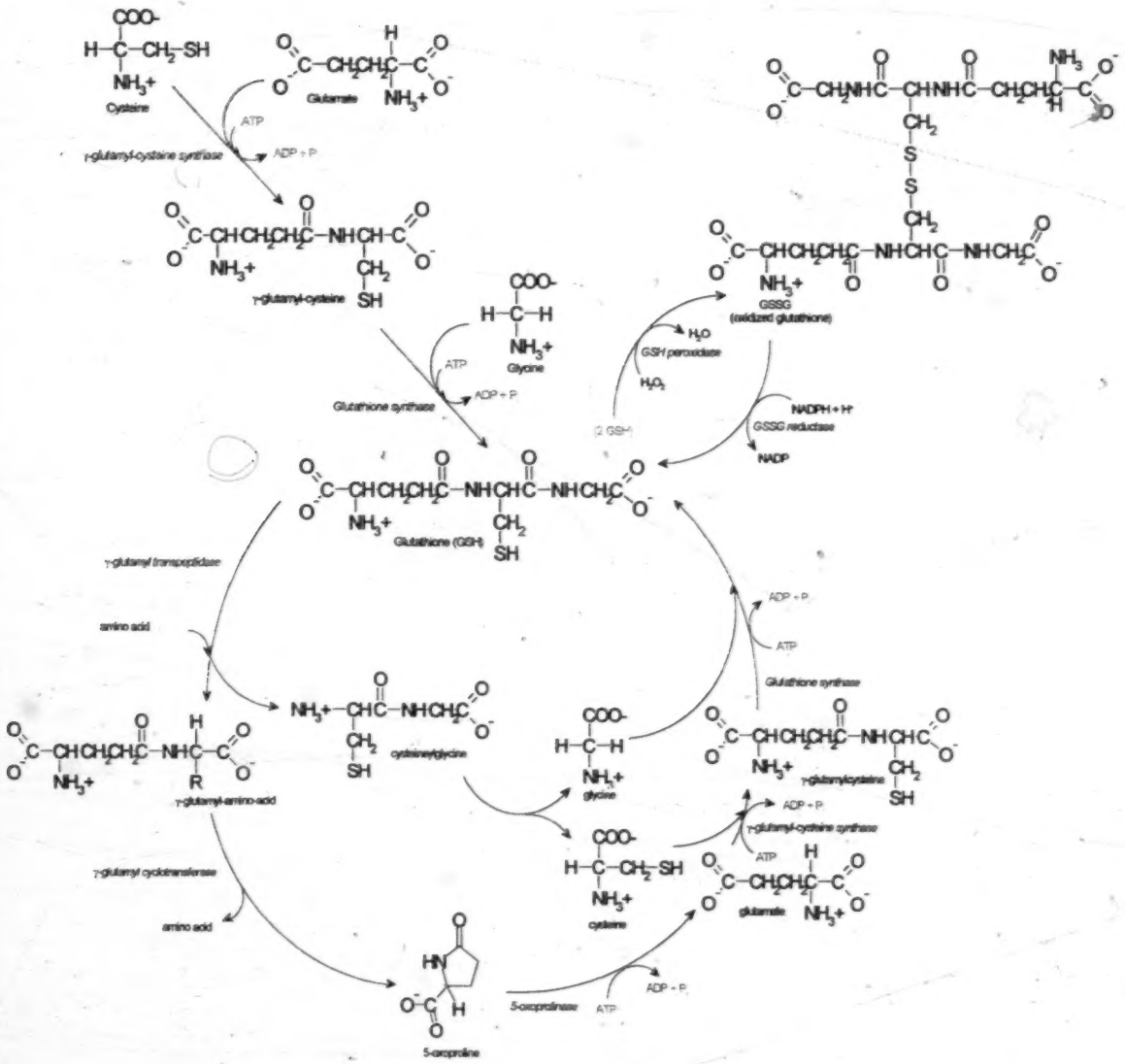
GSH is abundant in mucosal cells of the small intestine with concentrations higher in the proximal versus the distal portions of the small intestine (Loguercio and Di Pierro,

1999). GSH is secreted into the lumen from both the liver and from the mucosa (Bai and Jones, 1996) where it may serve to: aid in normal intestinal function and used to detoxify fatty acid hydroperoxides, maintain redox status, and regulate enzymes on the intestinal brush border. GSH may also be important for the absorption of iron and selenium (Dahm and Jones, 1994). GSH alters mucus fluidity via the thiol/disulfide ratio of GSH secreted into bile from the liver (Dahm and Jones, 2000).

When GSH synthesis is chemically inhibited there are alterations in the mucosal architecture of the small intestine resulting in intestinal lesions, inflammation and a relaxing of the junctions between enterocytes (Martenssen et al, 1990). Also, in cases of inflammatory bowel disease, increased GSH concentration attenuates acute colitis (Ardite

et al., 2000). At weaning, piglets experience anorexia, thus limiting the availability of the dietary precursors to GSH. This limitation coupled with the inflammation observed in the small intestine of piglets at this time (McCracken et al., 1999) suggests that GSH may play a vital role in maintaining gut health, structure and absorptive function. It remains unclear if excess cysteine results in increased mucosal GSH synthesis and if so, what affect this may have on the small intestine of the EW piglet.

Figure 2.2. Metabolic Pathway Associated with Glutathione (Adapted from Stipanuk et al., 1994)



3.0 Summary and Objectives

3.1 Summary and Rationale

At weaning, the structural, enzymatic and absorptive capacity of the gastro-intestinal tract is altered due to stress resulting from changes in environment and diet. In order to ameliorate the effects of weaning, diets are formulated to reduce the impact of reduced intake often observed at weaning (Alberta Agriculture, Food and Rural Development, 2000). However, many of the feedstuffs used in weaning diets, such as spray dried plasma, milk powder and whey are low in methionine (MET) and cystine (CYS_2) (NRC, 1998), resulting in MET becoming the first limiting amino acid in weaning diets. MET is an indispensable amino acid necessary for protein synthesis, methyl donation and cysteine (CYS) synthesis. CYS_2 is a conditionally dispensable amino acids required for protein, taurine and glutathione synthesis. Metabolically, the gastrointestinal tract (GIT) is a highly active organ. Recent research indicates that the GIT not only digests and absorbs nutrients, but also utilizes a larger percentage of these nutrients than previously believed (Rose, 1957; Wu, 1999; Stoll et al., 1998; Elango et al., 2002). Stoll et al. (1998) reported that approximately 52% of dietary MET disappears during first pass metabolism. Shoveller et al. (2003a) reported a 30% lower requirement for MET for intravenously fed as compared to gastrically fed piglets. It is currently unclear as to the fate of the dietary MET in the small intestine or, if MET or CYS_2 play a role in the gastrointestinal maturation of the early-weaned piglet.

NRC (1998) recommends that the total sulphur amino acid (TSAA) requirement for 3-5 kg piglets be 0.50 g/kg/d. However, the recommendation is not based on empirical evidence, rather it is extrapolated from data for heavier pigs. Recent evidence from Shoveller et al., (2003a) has determined, utilizing the indicator amino acid oxidation method, that the MET requirement is 0.42 g/(kg/d). Because MET can meet 100% of the TSAA requirement, this evidence indicates that the requirement for this weight class is actually 84% of the recommendation made by NRC (1998). In order to ensure that weaned piglets can achieve their maximum growth potential more empirical evidence is necessary to determine the requirement for the sulphur amino acids for 3.5 kg piglets.

CYS_2 has been reported to spare 20-70 % of the MET portion of the TSAA requirement (Chung and Baker, 1992b). NRC (1998) recommends a MET: CYS_2 ratio of 1:1. A typical wheat- soybean starter diet, contains 0.275 – 0.29 g/kg/d MET, and CYS_2 ranges from 0.35- 0.37 g/kg/d, approximately 125% greater than the MET inclusion. At weaning, piglets consume little if any feed and this reduced feed intake may be exacerbated if an amino acid imbalance exists. However, sows milk, the diet piglets are evolved to consume, contains a ratio of 2:1 (MET: CYS_2). The paucity of direct empirical evidence relating to the TSAA requirement in 5 kg pigs, the lack of information regarding the effect of excess dietary cystine in the piglet and ideal ratio of MET to CYS_2 for 3-5 kg piglets requires further investigation to determine the requirement and ratio of SAA that permits the optimisation of growth and gastrointestinal development in weaned piglets.

3.2 Hypothesis

1. Piglets receiving deficient, adequate or excess intakes of TSAA will have differences in growth and small intestinal development.
2. Piglets receiving different dietary ratios of MET to CYS_2 will have differences in growth and small intestinal development.

3.3 Objectives

In order to address the hypotheses stated above, the following objectives were developed:

1. Examine differences in growth, nitrogen retention and several small intestinal morphological and histological parameters in piglets receiving diets that meet: 50%, 75%, 100%, 150% and 200% of the total sulphur amino acid requirement recommended by NRC (1998).
2. Examine differences in growth, nitrogen retention and several small intestinal morphological and histological parameters in piglets receiving MET and CYS_2 at ratios of 1:1, 1:2 or 2:1.

4.0 Sulphur Amino Acid Supplementation in Early Weaned Piglets

4.1 Introduction

Early weaning is a management strategy utilized to increase the health status of pigs. Early weaning results in young piglets (<21 days) consuming solid diets that they are not fully capable of digesting. In an effort to increase digestibility and palatability of diets, nutritionists use feed ingredients that encourage consumption but unfortunately are low in methionine, an essential amino acid, and that have a MET: CYS_2 ratio that is the reverse of sow's milk. NRC (1998) recommends that MET be available to 3-5 kg piglets at 0.5 g/kg/d and be present in the diet in a ratio of 1:1 (MET: CYS_2). However, these recommendations are not based on direct empirical evidence but are instead extrapolations of growth curves for older pigs. Recent evidence indicates that the TSAA requirement for this weight class of pig is lower than the NRC (1998) recommendation (Shoveller et al., 2003a) and that the ratio of 1:1 MET: CYS_2 may not be ideal for growth and development (Shoveller et al., 2003b). An experiment was designed, using diets containing various concentrations and ratios of the sulphur containing amino acids, to investigate the requirement and ratio of the sulphur amino acids in early-weaned pigs.

4.2 Animals and Housing

The University of Alberta's Faculty Animal Policy and Welfare Committee approved all of the animal sampling procedures prior to the start of the experiment.

Forty-two male, castrated piglets (10 days of age and 3.5 kg ± 0.37 SD) were selected. The piglets were either purebred Genex Landrace (n=21) or crossbred stock (n=21, Landrace x Large White) and were for statistical necessity, blocked according to breed (f_1= Large White or f_2= crossbred). The piglets were weaned to the early weaning room at the University of Alberta Swine Research Centre (Building F-71) at 8:00 am on day 1. Piglets were paired in pens, 120 cm x 40 cm x 40 cm, on the basis of similar weight. Each pen had a trough water nipple and a removable 2-hole feeder. The pens were also equipped with a 35 cm x 30 cm barred window to allow piglets to have visual contact with other animals. A heat lamp was suspended above the dividing wall between two pens to provide supplemental heat for the piglets. This supplemental heat allowed for a gradation of temperature from 30°C under the heat lamp to 25°C at the front of the pen allowing piglets to determine their own thermal comfort zone. The lights were on a 12 h on/ 12 h off regimen.

Piglets were evaluated every day as a means of monitoring and evaluating their health and well-being. Piglet health and well-being were excellent throughout the experiment. Piglets were not allowed to intermingle but contact and line of sight was allowed through the barred window in each pen wall. This window allowed a limited degree of normal weaned piglet behaviour without jeopardizing individual measurements required for the trial. Toys were provided for environmental enrichment.

4.2.1 Daily Care

During the trial lights were turned on at 07:00 h. Each piglet was evaluated for health and vitality each day. The evaluation entailed a 5-minute observation per piglet during which the qualitative scale described above was utilised. Daily piglet care began with the removal of the feeders from each pen. The piglets were then removed and weighed individually and their weights recorded. At the start of the trial, during the three-day adaptation, piglets were offered 100 g of feed on day 1, 150 g on day 2 and 250 g on day 3. After adaptation piglets were offered 125 g of fresh feed at 07:00 h after the previous days feed had been removed, weighed and recorded.

Total urine was collected daily into 1 L plastic Erlenmeyer flasks containing 10 ml of orthophosphoric acid (85% v/v) to prevent bacterial growth by maintaining samples at pH 1. The total urine volume was weighed, recorded and a 150 ml sub-sample was taken and stored at –25°C for later nitrogen analysis.

At 19:00 h the feeders were removed, orts were weighed and recorded and 125 g of fresh feed was placed in the feeders. The pigs were observed again for 5-10 minutes to evaluate their health and vitality and the lights were turned off until 07:00 h the following morning.

To encourage the piglets to eat, they were paired according to weight for the first 3 days. Pairing assisted the newly weaned piglets to learn the location and purpose of the water nipple and feeder from each other. Piglets were also hand-fed every two

hours for the first three days of adaptation. On the morning of day 4, after weighing, piglets that had returned to weaning weight (weaning weight ± 100 g) were randomly assigned to one of the 7 test diets (n=6/diet). Following the daily care outlined previously, the piglets were maintained on the test diets for 7 days.

4.2.2 Blood Sampling

Blood (2 ml) was drawn from the pigs on days 0, 2, 4, and 6 of trial and on day 11 at necropsy. Sampling was accomplished via vena cava puncture using a 1.5-inch, 21-gauge vacu-needle into 3 ml vacutainer tubes. The blood was centrifuged at 5,000 rpm (Beckman J-6M/E Centrifuge, Palo Alt, Cal. U.S.A) for 10 minutes and the plasma stored at –80°C until analyses for plasma amino acids, urea nitrogen, and ammonia could be performed.

4.2.3 Diet

In order to examine effects of dietary concentration and ratios of MET and CYS_2 on the development and gastrointestinal tract of early-weaned piglets, diets that provided a range of total sulphur amino acids (TSAA) were formulated. The semi-purified diets were formulated to 110% of NRC (1998) recommendations for piglets weighing 3 kg, consuming 250 g/day, for all amino acid requirements excepting MET and CYS_2. The diets met or exceeded recommendations for all other nutrient requirements outlined in NRC (1998). The diets included a number of feedstuffs to ensure that the palatability of each diet would encourage the piglets to eat (Table 4.1). The TSAA ranged was from 50-200% of NRC. The 7 diets: a) 25-25 (0.25 g/kg/d), b) 25-50 (0.38 g/kg/d), c) 50-25 (0.38 g/kg/d), d) 50-50 (0.50 g/kg/d), e) 50-100 (0.75 g/kg/d), f) 100-50 (0.75 g/kg/d) and g) 100-100 (1.00 g/kg/d), (% MET-%CYS

as a % of NRC) (TSAA concentration of the diet) allowed investigation into the effects of feeding various TSAA concentrations and different ratios of methionine and cystine upon the growth and gastrointestinal development of the early-weaned piglet.

4.3 Necropsy

On the morning of the 11th day of the trial, piglets were removed from their pens and transported to the University of Alberta's Metabolic Research Centre for necropsy. The animals were rendered unconscious with Halothane at a flow rate of 5 ml/min and weighed. The duodenum was excised first after being identified from the exit of the stomach to the ligament of Trietz. The segment was removed and the lumen rinsed of its contents. The length was measured, weighed and two 2 cm segments were removed from the proximal end and either frozen instantly in liquid nitrogen or fixed in Formalin for protein analysis and histology respectively. The remaining small intestine (SI) was then excised and the total length and weight were measured and recorded. The SI was then sectioned into 4 equal parts; a) proximal jejunum, b) mid jejunum, c) distal jejunum and d) ileum. Of the four partitions, 85 cm from the mid-section of each partition was removed. The sampled sections were rinsed of lumenal contents and two 2 cm segments were removed for protein determination and histology. The remaining 80 cm of intestine was weighed and then gently scraped with a glass slide. The resulting mucosa was bagged, labelled, weighed and rapidly frozen in liquid nitrogen for subsequent amino acid concentration, protein determination and enzyme analysis. The liver, spleen and kidneys were removed and

weighed and 5 g samples of kidney and liver were taken and frozen in liquid nitrogen for amino acid concentrations. A 5 g sample of the longissimus dorsi was removed and frozen in liquid nitrogen for later intracellular amino acid profile analysis. The entire stomach was excised and the wall examined externally for ballooning or thinning of the lining. The stomach was then cut to reveal the interior for examination for ulceration and scoring. If an ulcer was present, it was scored and recorded otherwise the stomach was reported as normal. However, no ballooning or thinning was observed for any of the piglets in the experiment and was therefore not reported. The piglet carcass was then bagged with all of the remaining tissues and was stored at –80°C for later carcass analysis.

4.4 Carcass Grinding and Freeze Drying

Piglet carcasses, containing all tissues, barring those removed for analysis, were stored in a –80°C freezer until analyses. Frozen piglets carcasses were cut using a band saw, into small squares and then placed in a Hobart meat grinder. The carcasses were ground twice and mixed between grindings to ensure a thorough grinding and mixing of tissues. The grinder was washed with hot water and soap between each grinding to prevent cross -contamination of samples. After grinding, a 1 kg sample was removed and placed in a drum freeze drier (Virtronics, Gardnier, NY). After drying, the samples were removed and allowed to equilibrate with the atmosphere and the weight of the sample was measured and recorded. The samples were then placed in plastic containers, labelled and stored at –80°C until later analyses for protein and fat could be performed.

Table 4.1 Experimental Diet Composition (g/kg) as- fed

Ingredients	g/kg
Peas	50
Wheat	100
Whey	100
Spray Dried Plasma	25
Oat Groats	40
Dried Skim Milk Powder	150
Canola Oil	70
Vitamin/Mineral Premix[1]	40
Sugar	260
Choline	2
Aspartate[2]	70-80
Crystalline Amino Acid[3,4]	76.2-95.6
Dicalcium Phosphate	5
Chemical Analysis	
Metabolisable Energy MJ/kg	14.23
Crude Protein (g/kg)	240
Lysine (g/kg)	15

1 Supplied as above per kg of supplement: 300,000 IU Vitamin A, 3500 IU Vitamin D, 635 IU Vitamin E, 880 IU Vitamin B_{12} µg, 175 mg Riboflavin, 950 mg Niacin, 625 mg Pantothanoic acid, 6 µg Biotin, 80 µg Folacin, 7015 mg iron, 1600 mg manganese, 3470 mg zinc, 595 mg copper, 7.4 µg selenium

2 Aspartate was used to maintain the isonitrogenicity of the diets. As the TSAA concentration of the diets changed so too did the amount of aspartate used.

3 Amino acids (% of diet): Arginine: 0.55, histidine: 0.44, isoleucine: 0.75, leucine: 1.38, lysine: 1.37, phenylalanine: 0.82, tyrosine: 0.46, threonine: 0.86, tryptophan: 0.25, valine: 0.93

4 TSAA varied from 0.38% to 1.56% of the diet

4.5 Nitrogen Analysis

4.5.1 Kjeldahl

Urine nitrogen was analysed using a macro-Kjeldahl method based on method 976.05 (AOAC, 1990).

4.5.2 Feed and Carcass Nitrogen Analysis

Feed and carcass samples were analysed via LECO FP 428 (Leco Instruments LTD. Mississauga ON, Can) for nitrogen analysis. One hundred mg of sample were weighed, packaged in foil and combusted to determine the amount of nitrogen in each sample.

4.6 Amino Acid Profiles

4.6.1 Feed amino acid

Feed samples were analysed utilizing AOAC (1990) official method 994.12. Twenty-five mg of each feed underwent acid hydrolysis using sodium metabisulfite to protect the methionine and cystine in the samples, followed by performic acid oxidation. The oxidized samples were then derivatized utilising floraldehyde reagents prior to HPLC analysis (Varian 5000 HPLC and a Varian Flourichrom detector).

4.6.2 Plasma Amino Acids

Plasma amino acids were analysed via reverse phase HPLC utilizing a phenylisothiocyanate derivative as outlined by House et al. (1997).

4.6.3 Intracellular Amino acids

Two hundred mg of tissue was homogenized in 9 ml of 2% perchloric acid using a polytron (Brinkman Instruments. Rexdale, ON, Can). The resulting homogenate was centrifuged and the supernatant filtered through a 25 µm Millipore filter. The supernatant was derivatized following the same procedure as for the plasma amino acids (Bidlingmeyer et al., 1984).

4.7 Plasma Ammonia

Plasma ammonia was analysed using a diagnostic kit (Sigma number 171-UV). Plasma ammonia was calculated based on the conversion of 2-oxoglutarate to glutamate.

4.8 Enzyme Analysis

Mucosal enzymes were assayed to determine content and profile of disaccharide enzymes in piglet intestine. The assay was modified from Dahlqvist (1968), for individual cuvets, to a 96 well plate. 200 mg of mucosal samples were homogenized using a polytron grinder (Brinkmann Instruments. Rexdale. ON, Can) in 25 ml of distilled deionised (ddi) water. Samples were then centrifuged and the resulting supernatant was removed. Ten µl of unknown for lactose and maltose and 25 µl of unknown for sucrose was added to 22 µl of 0.1 M phosphate buffer pH 6.1 and 24 µl of either sucrose, lactose or maltose (0.058M) was added to the well. The larger volume was used for sucrose because Tris buffer inhibits sucrase activity. The

samples were then incubated for 30 min at 37°C. After the incubation, 242 µl of PGO (glucose oxidase, o-diaisidine and peroxidase) was added to the plate well and incubated for half an hour at 37°C. The plate was then read on a spectrometer (Molecular Devices Spectra Max 190, Sunnybrook, Ca. USA) at 470 nm and the absorbance compared against a glucose standard curve.

4.9 Protein Determination

Protein was analysed utilising a Bicinchonic Acid kit (Sigma, BCA-1). Samples were analysed utilizing the 96 well-plate method and were read on a spectrometer (Molecular Devices Spectra Max 190, Sunnybrook, Ca. USA) at 560 nm.

4.10 Histology

Formalin fixed tissues were sent to Department of Laboratory Animal Services, University of Alberta. Samples (n=126) were sectioned at 5 µm, and stained with Hematoxylin and Eosin using standard techniques. A Certified Veterinary Pathologist examined the slides. Observations were made on each section, and consisted of four separate measurements of the crypt depth and villus height, an assessment of the presence or absence of inflammatory changes, the morphology of villi, the presence/absence of hyperplasia and presence/absence of cells in the crypt lumens.

4.11 Protein Determination: Feed

One g samples of feed were compressed into pellets and analysed utilizing a LECO AC-300 bomb (Leco Instruments LTD. Mississauga, ON, Can).

4.12 NDF analysis

A pulverised feed sample (500 mg) was analysed for Neutral Detergent Fibre (Komarek, 1993). Briefly, the samples were placed into filter bags and heat-sealed. The bags were then placed in 100 ml/sample of neutral detergent solution. The solution was kept at 98°C for 70 minutes while the bags were agitated in the neutral detergent solution. After 70 minutes the bags were removed from the solution and rinsed 3 times with hot water (80-90°C). 0.05 ml/sample of amylase was added to the water. Bags were rinsed until the water no longer changed colour. The bags were then removed from the hot water and soaked for 3 minutes in acetone. The samples were then air-dried and placed in a 100°C oven over night. The samples were equilibrated with room temperature in a dessicator and then weighed. The equation: (final weight) - (filter bag weight)/(sample weight) * 100 = % fibre was used to calculate NDF.

4.13 Fat

Samples of carcass and feed underwent ether fat extraction utilizing the Goldfisch extraction apparatus (AOAC 920.39, 1990).

4.14 Moisture

Sample moisture was analysed by drying the feed at 60°C for 2 days (AOAC 930.15, 1990).

4.15 Statistical Analysis

The experiment was designed as a completely randomised trial. All of the datasets were analysed using SAS (SAS, V.6.06: SAS Institute, Cary, NC, USA) proc MIXED procedures. Dietary TSAA, MET, CYS intake and CYS(MET) interactions were used as main effects while the body weight of the piglet on day 3 of the trial, when experimental diets were introduced, was tested and used as a covariate for all growth datasets. Differences between means were tested using Dunnetts comparisons, the proc MIXED default, and considered significant at P values < 0.05. Trends were considered at $P < 0.10$.

4.16 Results

Throughout the trial all of the 42 animals were healthy and active and there was no requirement for the use of antibiotics as a medical treatment. Trends ($P<0.1, >0.05$) are mentioned in several places in this section because there was a consistent and important pattern in many of the results although not all achieved significance.

4.16.1 Growth

There were no significant differences ($P > 0.05$, Table 4.2) among the weights of the piglets on day 0, weaning weight. There were no significant differences ($P > 0.05$, Table 4.2) among the weights of the piglets on day 3, the beginning of experimental period. All of the animals gained weight over the course of the trial. There was an affect of TSAA intake on weight gain ($P = 0.004$, Table 4.2). Piglets receiving the deficient diets: 25-25 and 25-50 had significantly lower weights on day 10 of the trial ($P < 0.0001$, Table 4.3) compared to those animals receiving all other diets.

Table 4.2 Mean Weights (kg) at Weaning (Day 0), at the Start of Experimental Diets (Day 3) and on day 10, Average Daily Gain (ADG, g), Average Daily Feed Intake (ADFI, g) and Gain to Feed Ratios (G:F) of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Day 0[2,3]	3.88	3.55	3.98	3.75	3.77	0.14	N/A
Day 3[2,4]	3.83	3.52	3.88	3.70	3.81	0.16	N/A
Day 10[2,5,6]	4.44[a]	4.72[b]	5.03[c]	4.79[b]	4.83[b]	0.10	0.004
ADG[2,5,6]	81.67[c]	139.17[b]	187.52[a]	176.31[a]	129.52[b]	17.64	0.002
ADFI[2,5,6]	136.93[a]	161.67[b]	196.09[b]	179.31[b]	129.52[a]	15.76	0.07
G: F[2,5]	0.59[a]	0.85[b]	0.94[bc]	0.97[c]	0.73[bc]	0.07	<0.0001

1. Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values LSMEANS as calculated by Proc MIXED
3. Weight of piglets at weaning
4. Weight of piglets after 3 day adaptation
5. Letter differences within a row indicates significance (P<0.05, Dunnets)
6. ADG and ADFI used weight on day 3 as a covariate, weight on day 10 used day 3 wt and ADFI as a covariate

Table 4.3 Mean Weights (kg) at Weaning (Day 0), at the Start of Experimental Diets (Day 3) and on day 10, Average Daily Gain (ADG, g), Average Daily Feed Intake (ADFI, g) and Gain to Feed Ratios (G:F) of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100		P-value		
TSAA	50	75	75	100	150	150	200	Pooled SEM	MET	CYS	CYS(MET
n	6	6	6	6	6	6	6				
Day 0[2,3]	3.88	3.63	3.46	3.98	3.66	3.84	3.77	0.14	N/A	N/A	N/A
Day 3[2,4]	3.83	3.52	3.52	3.88	3.56	3.83	3.81	0.16	N/A	N/A	N/A
Day 10[2,5,6]	4.42[b]	4.56[b]	4.87[a]	5.03[a]	4.74[ab]	4.88[a]	4.83[a]	0.15	0.004	0.04	0.79
ADG[2,5,6]	81.67[c]	54.29[c]	185.56[a]	184.95[a]	171.00[a]	180.95[a]	129.88[b]	17.62	<0.0001	0.13	0.50
ADFI[2,5,6]	135.84[b]	127.99[b]	197.29[a]	195.91[a]	178.53[a]	180.81[a]	177.55[a]	15.28	<0.0001	0.54	0.85
G: F[2,5]	0.59[c]	0.73[b]	0.97[a]	0.94[a]	0.96[a]	0.96[a]	0.71[b]	0.07	0.005	<0.0001	<0.0001

1. Diets: methionine: cystine ratio relative to requirement (100%)
2. All values reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = interaction of cystine within methionine intake
3. Weight of piglets at weaning not on test diet.
4. Weight of piglets after adaptation to solid feed and prior to the start of experimental diets.
5. Letter differences within row indicates significance ($P < 0.05$, Dunnetts) for MET, CYS and CYS(MET)
6. ADG and ADFI used weight on day 3 as a covariate, weight on day 10 used day 3 wt and ADFI as a covariate

Figure 4.1 Average Daily Gain (ADG, g) on a Percent TSAA Requirement Basis for Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids

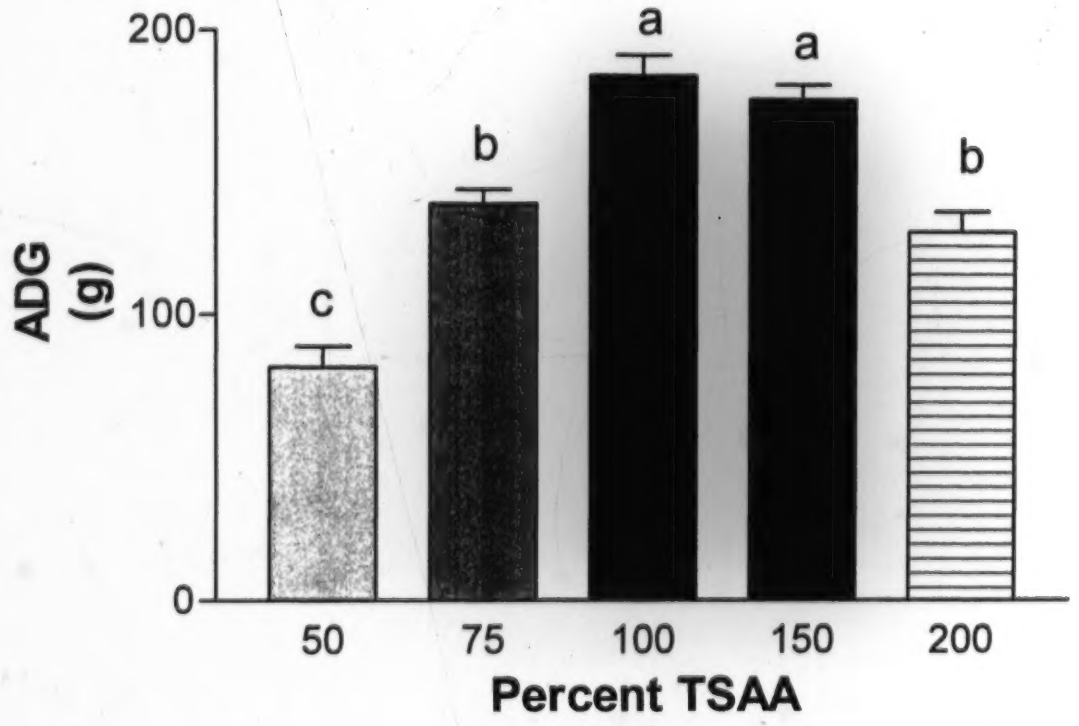


Table 4.4 Average Nitrogen Intake, Excretion, Retention (g Nitrogen/ day) and Balance (%) for Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
N Intake[2,3]	4.84	5.50	6.78	6.18	6.23	0.51	0.09
N Excretion[2,3,4]	1.35	1.22	1.24	1.15	1.34	0.14	0.70
N Retention[2,3,4]	3.39[b]	4.19[a]	5.52[a]	5.01[a]	4.89[a]	0.48	0.04
N Balance[2]	56[b]	76[a]	82[a]	80[a]	78[a]	7	0.04

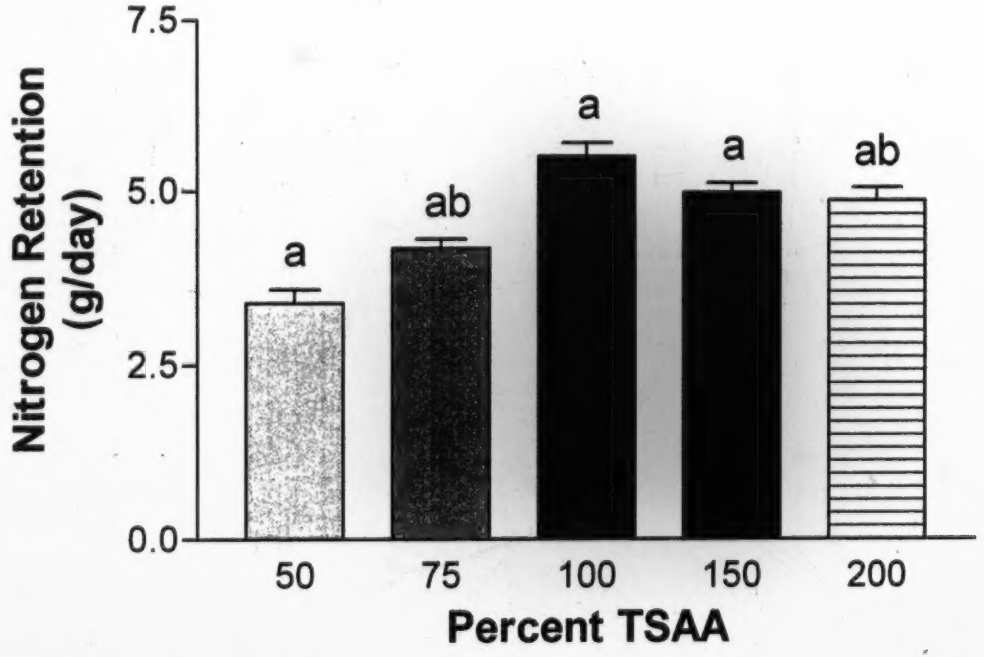
1 Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2 All values LSMEANS as calculated by Proc MIXED
3 Nitrogen retention and excretion used ADFI as a covariate
4 Letter differences within a row indicates significance (P<0.05, Dunnets)

Table 4.5 Average Nitrogen Intake, Excretion, Retention (g Nitrogen/ day) and Balance (%) for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-values		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
N Intake[2,3]	4.84	4.38	6.62	6.78	6.01	6.36	6.22	0.52	0.003	0.53	0.70
N Excretion[2,3,4]	1.49[a]	1.45[a]	1.09[b]	1.12[b]	1.03[b]	1.18[b]	1.29[b]	0.14	0.04	0.97	0.72
N Retention[2,3,4]	4.33[b]	4.34[b]	4.71[a]	4.72[a]	4.80[a]	4.63[a]	4.58[a]	0.17	0.05	0.86	0.81
N Balance[2]	56	70	81	81	81	78	78	7	0.27	0.50	0.49

1. Diet: methionine: cystine relative to requirement (100)
2. All values reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3. Nitrogen excretion and retention used ADFI as a covariate
4. Letter differences within a row indicates significance ($P<0.05$, Dunnetts) for MET, CYS and CYS(MET)

Figure 4.2 Nitrogen Retained (g/day) on a Percent TSAA Requirement Basis of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids



Both MET (P= 0.004, Table 4.3) and CYS (P = 0.04, Table 4.3) concentrations of the diet affected weight on day 10. As dietary MET intake increased so too did the weight of the piglets.

TSAA intake significantly affected average daily gain (ADG) (P = 0.002, Table 4.2). As TSAA intake increased to requirement so too did ADG. As TSAA intake exceeded requirement ADG decreased. Piglets receiving diets 25-25, 25-50 and 100-100 had significantly lower rate of gain than piglets receiving diet 50-25, 50-50, 50-100 and 100-50 (Table 4.3), as illustrated in Figure 4.1. Piglets receiving diet 100-100 had a significantly lower ADG (P = 0.02, Table 4.3) than those receiving 50-25, 50-50, 50-100, 100-50. MET intake significantly affected ADG of the pigs during the course of the trial (P <0.0001, Table 4.3). As MET intake increased to 50% of the TSAA requirement a concurrent increase in ADG occurred. There was no effect of increasing dietary CYS_2 intake on the ADG of the piglets.

There was a trend for TSAA intake to effect average daily feed intake (ADFI) (P=0.07, Table 4.2). As TSAA intake increased to requirement, ADFI increased, but for intakes beyond requirement ADFI decreased. Piglets consuming diets 25-25, 25-50 and 100-100 consumed the least diet (P <0.0001, Table 4.3). ADFI was significantly affected by MET intake. When MET intake was at 50% of the TSAA requirement ADFI intake increased and remained constant with further rises in MET intake. There was no effect of dietary CYS_2 intake on ADFI.

Over the entire experimental period (7 days) TSAA, MET and CYS intake ($P < 0.0001$, $P = 0.005$, $P < 0.0001$, Table 4.2 and Table 4.3 respectively). As MET concentration of the diet increased from 25% to 50% of NRC so too did the gain efficiencies of piglets but no improvement was observed as MET intakes rose from 50% to 100% of NRC, however at 200% of the TSAA requirement piglets were less efficient.

4.16.2 Nitrogen Balance

There was a trend for TSAA intake ($P = 0.09$, Table 4.4) to affect the intake of nitrogen by the piglet. As the TSAA intake increased to requirement, intake increased and reached a plateau. There was an effect of MET ($P = 0.003$, Table 4.5) on nitrogen intake. As dietary MET intake rose to 50% of NRC requirement so too did nitrogen intake, further increases of MET concentration did not result in greater intakes of nitrogen. There was no effect of dietary CYS_2 intake on piglet nitrogen intake ($P > 0.05$, Table 4.5).

MET intake had a significant effect ($P = 0.04$, Table 4.5) on nitrogen excretion. Piglets consuming diet 25-25 and 25-50 excreted significantly greater amounts of nitrogen than the piglets consuming the remaining 5 diets, however as MET intake increased beyond 50% of the TSAA requirement so too did nitrogen excretion.

TSAA intake significantly effected nitrogen retention ($P = 0.04$, Table 4.4, Figure 4.2). As TSAA intake increased to requirement there was an increase in the amount

of nitrogen retained by the piglet, once at requirement retained nitrogen plateaued. There was a significant effect of MET intake on nitrogen retention ($P = 0.05$, Table 4.5). Piglets consuming deficient concentrations of MET (diets: 25-25 and 25-50) retained the least amount of nitrogen.

TSAA intake significantly affected nitrogen balance ($P = 0.04$, Table 4.4). Nitrogen balance increased as TSAA intake increased to TSAA requirement. However, no further increases occurred after requirement was exceeded. While there was no effect of MET or CYS_2 intake on nitrogen balance, animals consuming diets 25-25 and 25-50 had numerically lower balances than the piglets consuming the remaining diets. Piglets consuming diet 50-25 performed identically as those consuming diets 50-50, 50-100, 100-50 and 100-100.

There was a significant effect of TSAA intake ($P < 0.0001$, $P = 0.007$, Table 4.6) on plasma urea nitrogen on days 6 and 10 respectively. The effect of TSAA intake on plasma urea nitrogen was driven by the significant effect ($P < 0.0001$, $P = 0.005$, Table 4.7) of MET intake on plasma urea nitrogen on days 6 and 10. The piglets that received TSAA-deficient diets 25-25 and 25-50 had higher plasma urea nitrogen concentrations as compared to the piglets consuming the other diets. Once TSAA requirement as recommended by NRC (1998) was reached plasma urea nitrogen decreased and plateaued. On days 0, 2 and 4 there was no significant effect of MET or CYS_2 intake on plasma urea nitrogen ($P > 0.05$, Table 4.7).

4.16.3 Necropsy

TSAA intake significantly affected liver mass (P = 0.04, Table 4.8), liver mass corrected for body weight (P = 0.05, Table 4.8), kidney mass (P = 0.004, Table 4.8), kidney mass corrected for body weight (P = 0.05, Table 4.8) mass. As TSAA intake increased so too did the mass of the liver and kidney, however when TSAA concentrations was at 200% of NRC (1998) the weight of the liver decreased. There was no effect of TSAA intake on the spleen either on an absolute basis or when corrected for body weight. MET intake had a significant effect on liver and kidney mass (P = 0.007, P = 0.01, respectively, Table 4.9). There was a trend for MET (P = 0.09, Table 4.9) and CYS_2 intake (P = 0.10, Table 4.9) to affect the mass of the spleen. When organ mass was corrected for body weight, there was a trend (P = 0.07, Table 4.9) for the liver mass to be affected by MET intake but not the kidney or spleen.

TSAA intake had no effect on the small intestine (P > 0.05, Table 4.10). MET intake significantly affected (P = 0.02, Table 4.11) small intestine (SI) weight. MET intake also had a significant affect (P = 0.01, Table 4.11) on the length of the SI. While not significant, as dietary CYS_2 concentration increased there was a decrease in the weight and length of the small intestine, this pattern of increasing dietary CYS_2 to negatively impact intestinal parameters was observed repeatedly in most of the datasets.

Table 4.6 Plasma Urea Nitrogen (mmol/L) for days 0, 2, 4, 6 and 10 from Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Day 0[2,3]	2.99	3.12	2.99	3.13	2.93	0.37	N/A
Day 2	3.87	4.23	3.86	3.16	3.84	0.65	N/A
Day 4	3.58	3.72	3.05	3.45	4.01	0.49	0.42
Day 6[4]	5.90[a]	5.18[a]	3.47[b]	2.64[c]	3.40[b]	0.40	<0.0001
Day 10[4]	3.97[a]	3.38[a]	2.19[b]	2.06[b]	2.42[b]	0.53	0.007

1. Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. Day 0 (pre-weaning) and day 2 (on adaptation diet) expressed as an average with pooled SEM
3. All values reported as LSMEANS as calculated by Proc MIXED
4. Letter differences within a row indicates significance ($P<0.05$, Dunnets)

Table 4.7 Plasma Urea Nitrogen (mmol/L) for days 0, 2, 4, 6 and 10 from Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1] TSAA	25-25 50	25-50 75	50-25 75	50-50 100	50-100 150	100-50 150	100-100 200	Pooled SEM	P-value		
									MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Day 0[2,3]	2.99	2.81	3.10	3.13	3.05	3.19	3.02	0.37	N/A	N/A	N/A
Day 2	3.87	4.94	3.51	3.86	3.60	2.72	3.84	0.65	N/A	N/A	N/A
Day 4	3.58	3.78	3.67	3.05	3.66	3.23	4.01	0.49	0.59	0.28	0.50
Day 6[4]	5.99[a]	6.07[a]	3.96[b]	3.54[b]	2.66[c]	2.75[bc]	3.49[b]	0.40	<0.0001	0.49	0.07
Day 10[4]	3.97[a]	3.89[ab]	2.86[ab]	2.19[b]	2.11[b]	2.00[b]	2.42[b]	0.53	0.005	0.59	0.63

1 Day 10[3,4] Diet: methionine: cystine relative to requirement (100)
2 Day 0 (pre-weaning) and 2 (on adaptation diet) expressed as averages with pooled SEM
3 All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
4 Letter differences within a row indicates significance (P<0.05, Dunnetts) for MET, CYS and CYS(MET)

There was no effect of TSAA intake on the weight of the sampled small intestinal segments ($P > 0.05$, Table 4.12). There was no effect of MET or CYS_2 intake upon the mass of the duodenum or proximal jejunum ($P > 0.05$, Table 4.13). There was a significant effect of increasing dietary MET intake ($P = 0.02$, Table 4.13) on medial jejunal mass. Within a MET intake group (i.e. 25% MET, 50% MET and 100% MET) as dietary CYS_2 intake was increased, a decrease in mass was observed (Illustrated in Figure 4.4). There was a trend for differences in the weights of the medial ileum ($P = 0.08$, Table 4.13), as dietary MET intake increased so too did the weight of the medial ileum.

TSAA intake had no effect on the weight of the mucosa of the sampled small intestinal segments ($P > 0.05$, Table 4.14). Similarly, there was no effect of MET or CYS_2 intake ($P > 0.05$, Table 4.15) upon the mucosal mass of the duodenum or proximal jejunum. There was an effect of MET ($P = 0.02$, Table 4.15) and CYS_2 ($P = 0.02$, Table 4.15) intake on the weight of the medial jejunal mucosa. As dietary MET intake increased so too did the weight of the mucosa (Illustrated in Figure 4.5), however within a MET group as dietary CYS_2 intake increased mucosal mass was negatively affected. MET intake had a significant effect ($P = 0.03$, Table 4.15) on the mucosal mass of the medial ileum. While not significant, as dietary CYS_2 intake within a MET group increased, mucosal mass for the medial ileum decreased numerically.

There was no effect of TSAA intake on the mucosal weight/length of the sampled small intestinal segments ($P > 0.05$, Table 4.16). There was no effect of MET or CYS_2 intake on the mucosal weight/length of the duodenum ($P > 0.05$, Table 4.17). There was a trend ($P = 0.10$, Table 4.17) for increasing dietary MET intake to affect mucosal weight/length of the proximal jejunum. Both MET ($P = 0.02$, Table 4.17) and cystine ($P = 0.02$, Table 4.17) had an effect on the weight/length of the medial jejunum. As MET intake increased so too did the mucosal weight/length, and within a MET group as dietary CYS_2 intake increased mucosal weight/length decreased. MET intake had a significant effect ($P = 0.02$, Table 4.17) on the mucosal weight/length of the medial ileum. As intake of MET increased so too did the mass/length of the ileum. CYS_2 intake tended ($P = 0.10$, Table 4.17) to affect the weight/length of the medial ileum. As dietary CYS_2 intake within a MET group increased, the ileum was negatively affected.

4.16.4 Histology

TSAA intake had no effect on the crypt depth of the duodenum, jejunum or ileum ($P > 0.05$, Table 4.18). There was no effect of either MET or CYS_2 intake on the crypt depth of the duodenum or medial jejunum ($P > 0.05$, Table 4.19). MET intake had a significant effect ($P = 0.02$, Table 4.19) on the crypt depth in the medial ileum. As MET intake increased to 50% of the TSAA requirement, the crypt depth in the medial ileum deepened.

Table 4.8 Organ weight (g) and (g/kg BW) of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Liver[2,3,4]	123.10^{ab}	119.82^{ab}	114.54^{b}	131.30^{a}	131.63^{a}	6.62	0.04
Liver[2] (g/kg BW)	25.80^{ab}	25.04^{bc}	24.34^{c}	27.47^{a}	27.60^{a}	1.09	0.05
Kidney[2,3,4]	24.04^{c}	27.20^{c}	29.96^{b}	30.82^{a}	31.82^{a}	1.67	0.004
Kidney[2] (g/kg BW)	5.65^{b}	5.84^{ab}	5.96^{a}	6.23^{a}	6.59^{a}	0.31	0.05
Spleen[2,3,4]	9.94	11.59	10.49	11.34	11.35	1.26	0.63
Spleen[2,4] (g/kg BW)	2.38	2.39	2.17	2.28	2.38	0.25	0.90

1. Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values represented as LSMEANS as calculated by Proc MIXED
3. Letter differences within a row indicates significance ($P<0.05$, Dunnets)
4. Liver, Kidney and spleen mass used body mass on day 3 as a covariate

Table 4.9 Organ weight (g) and (g/kg BW) of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1] TSAA	25-25 50	25-50 75	50-25 75	50-50 100	50-100 150	100-50 150	100-100 200	Pooled SEM	P-value		
									MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Liver[3]	110.56[bc]	105.60[c]	127.84[ab]	121.73[bc]	132.23[a]	139.95[a]	133.60[a]	6.62	0.007	0.81	0.36
Liver (g/kg BW)	25.80	24.23	25.85	24.34	27.09	27.86	27.60	1.09	0.07	0.31	0.34
Kidney[3]	24.04[b]	25.86[ab]	28.54[ab]	29.97[ab]	30.62[a]	31.02[a]	31.87[a]	1.67	0.01	0.44	0.99
Kidney (g/kg BW)	5.65	5.92	5.76	5.96	6.25	6.21	6.59	0.31	0.43	0.18	0.97
Spleen[3]	9.85	10.74	12.68	10.36	13.00	9.68	11.28	1.26	0.09	0.10	0.19
Spleen (g/kg BW)	2.37	2.36	2.41	2.17	2.58	1.97	2.38	0.25	0.34	0.14	0.83

1. Diet: methionine: cystine relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine group
3. Letter differences within a row indicate significance ($P < 0.05$, Dunnetts) for MET, CYS and CYS(MET)
4. Liver, kidney and spleen used body weight on day 3 as a covariate.

Table 4.10 Small Intestinal (SI) Measurements of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Small Intestine[2,3] (g)	154.54	171.91	178.38	185.25	176.33	12.98	0.45
Small Intestine Length (cm)	879.86	920.09	898.48	928.30	908.62	26.33	0.62
Small intestine weight/ length (g/cm)	0.18	0.19	0.20	0.20	0.19	0.01	0.59

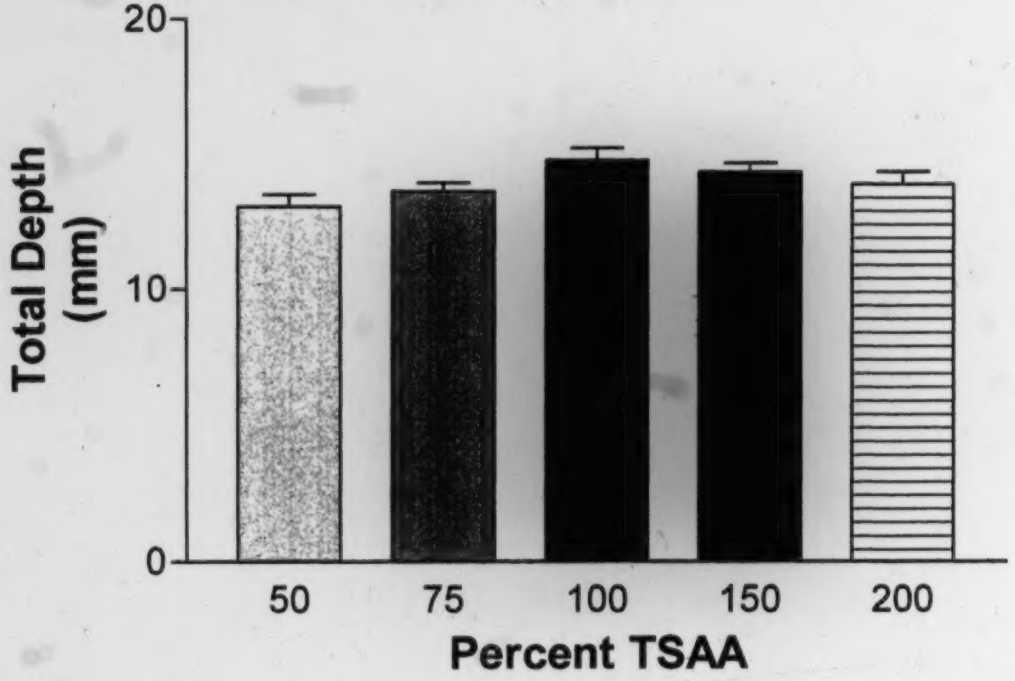
1. Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values reported as LSMEANS as calculated by proc MIXED
3. Small intestine weight and length used body weight on day 10 as a covariate

Table 4.11 Small Intestinal (SI) Measurements of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Small Intestine[2,3,4] (g)	154.28^{ab}	147.88^{b}	198.45^{a}	176.91^{ab}	183.81^{a}	186.81^{a}	176.12^{ab}	12.98	0.02	0.70	0.79
Small Intestine Length (cm)	880.93^{b}	868.88^{b}	960.58^{a}	911.12^{ab}	909.34^{ab}	947.02^{a}	909.51^{ab}	26.33	0.01	0.19	0.66
Small intestine weight/ length (g/cm)	0.17	0.17	0.20	0.19	0.20	0.19	0.19	0.01	0.18	0.98	0.90

1 Diet: methionine: cystine ratio relative to requirement (100)
2 All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3 Small Intestine weight and length used body weight on day 10 as a covariate
4 Letter differences within a row indicate significance ($P < 0.05$, Dunnetts) for MET, CYS and CYS(MET)

Figure 4.3 Total Depth (mm) of Mucosa on a Percent TSAA Basis for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids



There was a trend for TSAA intake (P = 0.09, Table 4.20) to affect the total mucosal depth of the medial jejunum. As intake reached TSAA requirement the total depth of the mucosa increased, however, as TSAA concentration of the diet increased beyond requirement the mucosal depth decreased. MET intake had a significant effect on the total mucosa depth of the duodenum (P = 0.05, Table 4.21), medial jejunum (P = 0.01, Table 4.21) and medial ileum (P= 0.004, Table 4.21). In the medial jejunum and ileum, the pattern of increasing dietary cystine intake within a methionine group decreased the total depth of the mucosa was observed.

4.16.5 Body Composition

There was a significant effect of TSAA intake on the moisture (P=<0.0001, Table 4.22) of the piglet. As TSAA intake increased so too did the moisture content. There was also a trend for TSAA intake to affect the crude protein percentage (P=0.07, Table 4.22) of the piglet. As TSAA intake reached requirement there was an increase in the amount of protein in the carcass, however, as TSAA concentration of the diet increased beyond requirement the amount of protein in the carcass decreased. There was a trend for MET intake (P = 0.09, Table 4.23) to affect the crude protein content.

4.16.6 Plasma Amino Acids

Circulating concentrations of alanine (P = 0.01, Table 4.24), arginine (P = 0.04, Table 4.24), glutamate (P = 0.02, Table 4.24) and taurine (P <0.0001, table 4.24) increased as TSAA intake increased. Circulating glycine (P = 0.003, Table 4.24) decreased as TSAA intake increased. TSAA intake tended to increase circulating hydroxyproline

(P = 0.08, Table 4.24) and decrease circulating tyrosine (P = 0.07, Table 4.24). MET and CYS_2 intake had no affect on the concentration of any of the circulating indispensable amino acids except histidine (P = 0.003, P = 0.002, Table 4.25). Increasing CYS_2 intake resulted in decreased histidine concentration. MET intake (P = 0.05, Table 4.25) increased plasma alanine concentrations. MET intake increased plasma hydroxyproline (P = 0.02, Table 4.25), serine (P = 0.03, Table 4.25) and taurine (P = 0.02, Table 4.25) concentrations. Increasing CYS_2 intake decreased plasma glycine (P = 0.01, Table 4.25) and increased taurine (P = 0.003, Table 4.25) concentrations.

4.16.7 Liver Amino Acids

TSAA intake increased hepatic proline (P <0.0001, Table 4.26), serine (P = 0.03, Table 4.26) and taurine (P = 0.04, Table 4.26). TSAA intake tended to increase methionine (P = 0.09, Table 4.26), phenylalanine (P = 0.09, Table 4.26) and arginine (P = 0.09, Table 4.26) and decrease threonine (P = 0.10, Table 4.26). There was no effect of MET or CYS_2 intake on the intra-cellular concentrations of the hepatic indispensable amino acids (P >0.05, Table 4.27). There was a trend (P = 0.06, Table 4.27) for increasing dietary MET intake to decrease hepatic threonine concentrations. Increasing MET intake increased hepatic intra-cellular arginine (P = 0.005, Table 4.27) and glycine (P = 0.02, Table 4.27). Increasing MET intake tended to increase hepatic proline (P = 0.10, Table 4.27) and serine (P = 0.10, Table 4.27) concentrations. Within a MET group increasing CYS_2 intake increased hepatic proline (P = 0.003, Table 4.27) and taurine (P = 0.02, Table 4.27) concentrations.

4.16.8 Kidney Amino Acids

TSAA intake increased renal isoleucine (P = 0.05, Table 4.28), lysine (P = 0.04, Table 4.28), alanine (P = 0.03, Table 4.28) and taurine (P = <0.0001, Table 4.28). As TSAA intake rose to requirement renal methionine (P <0.0001, Table 4.28) decreased, as TSAA intake exceeded requirement renal methionine concentration increased. MET intake increased (P = 0.04, Table 4.29) renal lysine concentrations. As MET intake increased renal arginine (P = 0.04, Table 4.29) concentration rose and as MET intake exceeded 50% of NRC requirement renal arginine decreased. As MET intake increased so too did renal glutamine (P = 0.01, Table 4.29) concentrations. Increasing MET intake tended to increase renal valine (P = 0.09, Table 4.29) and alanine (P = 0.08, Table 4.29). Within a MET group increasing CYS_2 intake increased renal taurine concentrations (P = 0.003, Table 4.29). Within a MET group increasing CYS_2 intake tended to decrease renal isoleucine (P = 0.08, Table 4.29), methionine (P = 0.10, Table 4.29), phenylalanine (P = 0.06, Table 4.29) and alanine (P = 0.06, Table 4.29).

4.16.9 Muscle Amino Acids

Increasing TSAA intake tended to increase muscular taurine (P = 0.06, Appendix 8) intra-cellular concentrations. None of the amino acid concentrations of the muscle tissues were affected by MET or CYS_2 intake. However, MET intake tended (P > 0.05, Appendix 8) to increase lysine muscular intra-cellular concentrations.

4.16.10 Protein and Enzymes

4.16.10.1 Mid Jejunal Protein Content

There was no effect of TSAA, MET or CYS_2 intake on the protein content of the mid jejunum ($P>0.05$, Table 4.30, Table 4.31). However, the protein concentration reflected the same pattern as seen in other small intestinal datasets. As the concentration of CYS_2 in the diet increases there was a non significant decrease in the protein concentration.

4.16.10.2 Enzyme Analysis

There was no significant effect of TSAA, MET or CYS_2 intake on the enzymatic profile of the mid jejunal sections of the small intestine ($P>0.05$, Table 4.32 and Table 4.33 respectively). However as CYS_2 intake increased there is a non-significant increase in maltase content.

4.17 Discussion

Early weaning has become a popular management strategy in swine production. The resulting weaned pig is on average 14-18 days old, substantially younger and less gastro-intestinally prepared to consume commercial weaning diets than their traditionally weaned (> 21 days) counterparts. As such, early-weaned piglets have specialized nutritional requirements in order to maximize growth potential. NRC (1998) makes recommendations for amino acid requirements for this weight class of piglet however, these recommendations were validated using only a small number of studies with various diet formulations and management strategies. These

Figure 4.4. The Weight of 80 cm of the Medial Jejunum of Piglets that Consumed Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

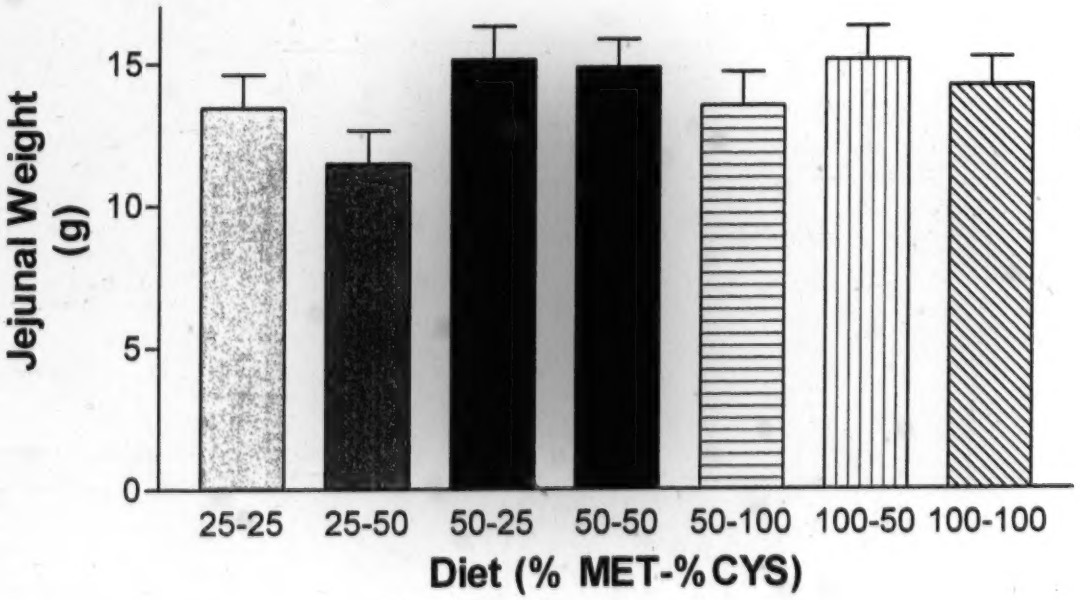


Figure 4.5. Weight (g) of Mucosa Recovered from 80 cm of the Medial Jejunum of Piglets that Consumed Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

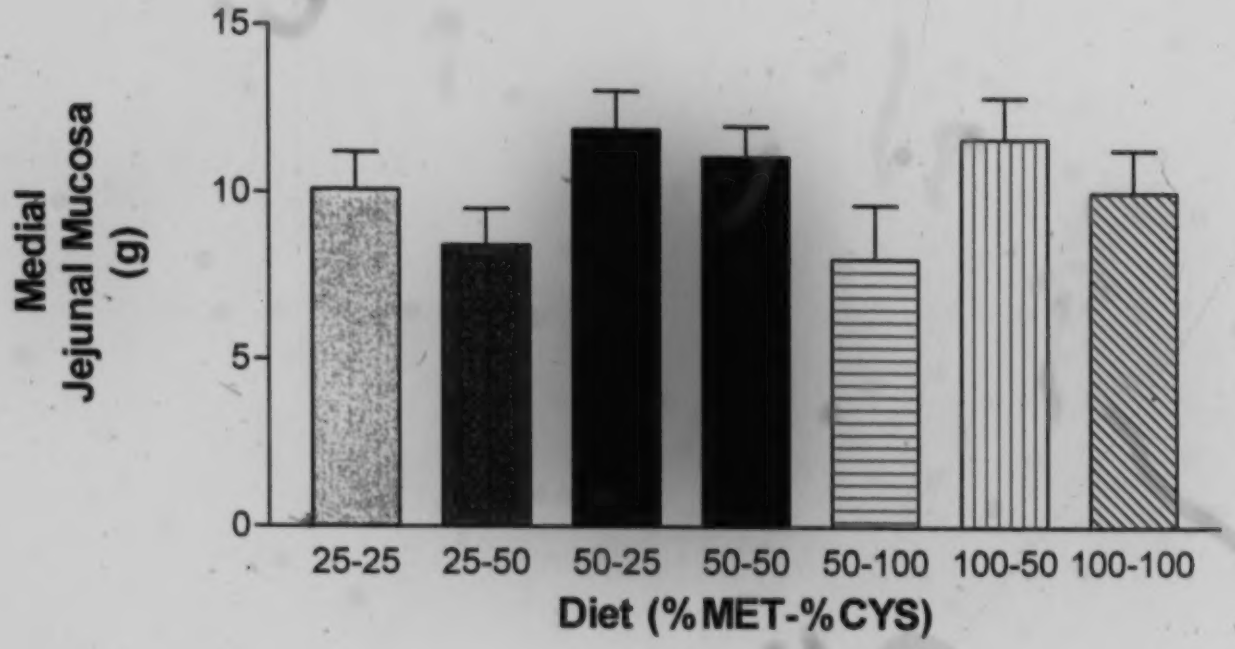


Figure 4.6. Weight (g) of Jejunal Mucosa Per Unit Length of Piglets that Received Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

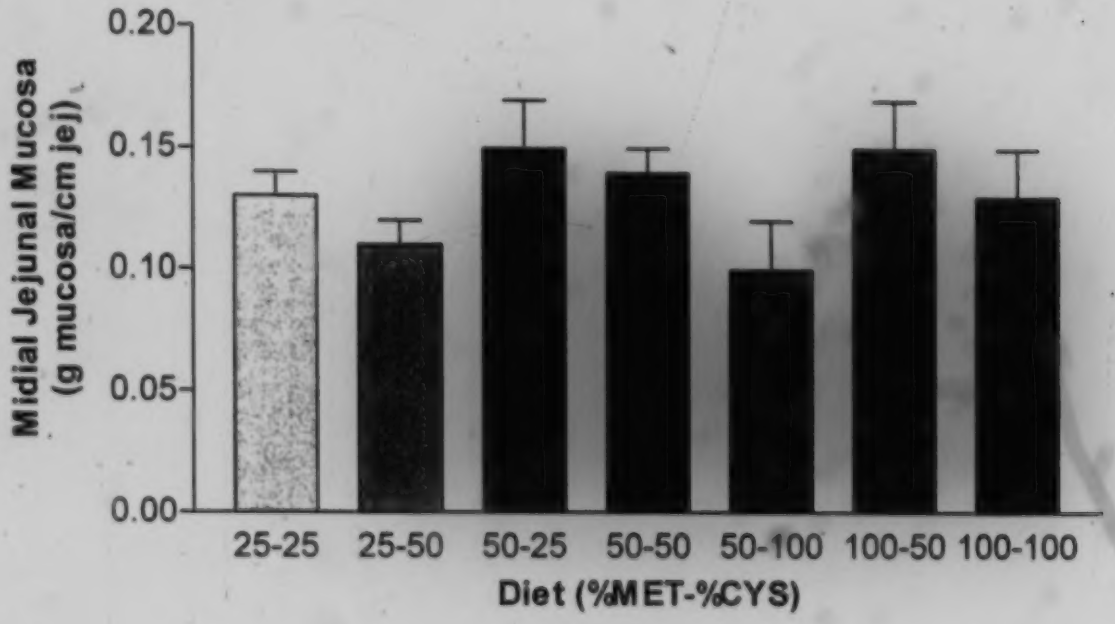


Figure 4.7. Total Depth of Medial Jejunal Mucosa (mm) of Piglets After Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

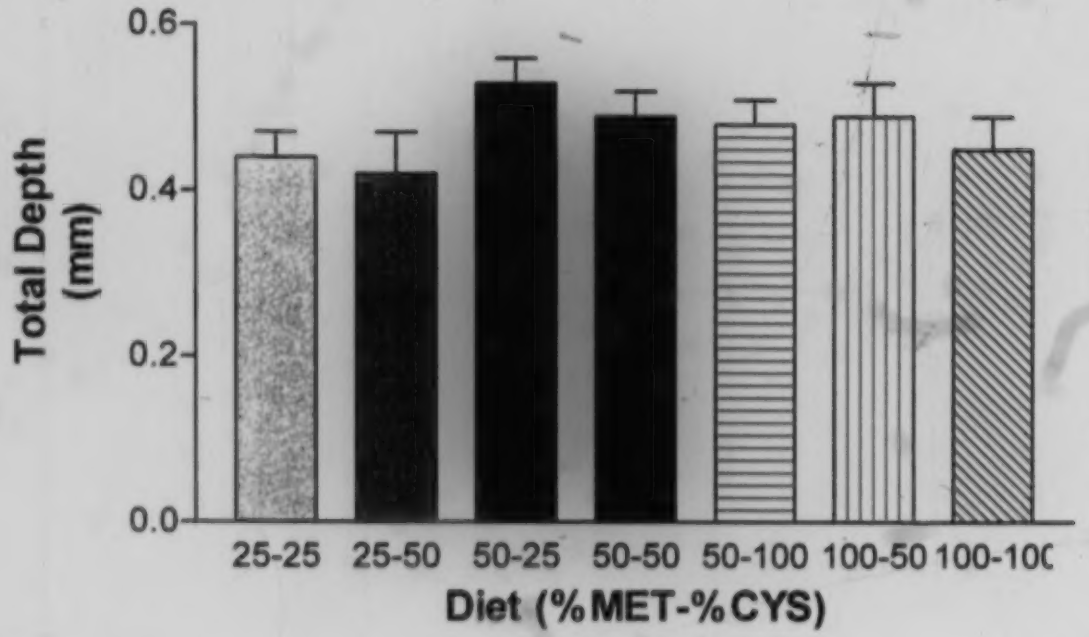


Table 4.12 Weights (g) of Intestinal Segment (80cm) from Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	11	6	12	6		
Duodenum[2]	3.61	3.80	4.19	3.68	4.48	0.26	0.15
Proximal Jejunum	12.64	13.65	16.59	13.91	14.43	1.19	0.23
Medial Jejunum	13.07	13.66	14.82	14.40	13.95	1.12	0.81
Medial Ileal	14.40	14.85	17.25	16.38	17.85	1.86	0.22

1. Diet represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values represented as LSMEANS as calculated by proc MIXED

Table 4.13 Weights (g) of Intestinal Segment (80cm) from Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-values		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	5	6	6	6	6	6				
Duodenum[2]	3.61	3.61	4.02	4.18	3.86	3.50	4.46	0.29	0.52	0.57	0.10
Proximal Jejunum	12.66	13.17	14.06	16.61	13.43	14.38	14.44	1.34	0.29	0.49	0.53
Medial Jejunum[3]	13.45^{ab}	11.48^{b}	15.15^{a}	14.88^{a}	13.55^{a}	15.16^{a}	14.27^{a}	1.18	0.02	0.30	0.97
Medial Ileal	14.40	13.78	15.92	17.25	15.67	17.10	17.85	1.96	0.08	0.98	0.59

1. Diet: methionine : cystine ratio relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET)= cystine intake within a methionine intake
3. Letter differences within a row indicate significance (P>0.05, Dunnetts) for MET, CYS and CYS(MET)

Table 4.14 Weight of Mucosa (g) Recovered From Small Intestinal Segments (80 cm) of Piglets that Consumed Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Duodenal Mucosa[2]	1.87	1.71	1.95	1.70	1.76	0.17	0.84
Proximal Jejunal Mucosa	9.27	10.43	11.45	10.99	10.79	1.19	0.69
Medial Jejunal Mucosa	9.59	10.63	11.03	9.92	9.66	1.07	0.84
Medial Ileal Mucosa	10.83	10.46	12.05	11.62	11.34	1.11	0.76

1. Diets represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values represented as LSMEANS as calculated by proc MIXED

Table 4.15 Weight of Mucosa (g) Recovered From Small Intestinal Segments (80 cm) of Piglets that Consumed Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Duodenal Mucosa[2]	1.87	1.57	1.93	1.93	1.72	1.67	1.76	0.19	0.60	0.61	0.60
Proximal Jejunal Mucosa	9.33	9.54	11.18	11.62	9.97	12.24	10.84	1.32	0.12	0.41	0.99
Medial Jejunal Mucosa[3]	9.61[ab]	8.54[b]	12.69[a]	11.04[ab]	8.62[b]	11.22[ab]	9.68[ab]	1.07	0.02	0.02	0.84
Medial Ileal Mucosa	10.98[a]	9.08[b]	12.26[a]	12.05[a]	10.30ab	12.95[a]	10.08[ab]	1.07	0.03	0.18	0.72

1 Diet: methionine: cystine ratio relative to requirement (100)

2 All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

3 Letter differences within a row indicates significance (P <0.05, Dunnetts) for MET, CYS and CYS(MET)

Table 4.16 Mucosa weight/ Length of Small Intestinal Segments (g/cm) from Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Duodenum[2]	0.09	0.09	0.11	0.09	0.09	0.03	0.27
Proximal Jejunum	0.12	0.13	0.15	0.14	0.14	0.02	0.68
Medial Jejunal	0.12	0.13	0.14	0.13	0.12	0.02	0.85
Medial Ileal	0.13	0.13	0.15	0.14	0.15	0.02	0.84

1. Diets represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED

Table 4.17 Mucosa weight/ Length of Small Intestinal Segments (g/cm) from Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Duodenum[2]	0.09	0.08	0.10	0.11	0.09	0.08	0.09	0.01	0.14	0.55	0.21
Proximal Jejunum	0.12	0.12	0.14	0.14	0.12	0.15	0.14	0.02	0.10	0.40	0.98
Medial Jejunal	0.13^{ab}	0.11^{b}	0.15^{a}	0.14^{a}	0.12^{b}	0.15^{a}	0.13^{ab}	0.01	0.02	0.02	0.85
Medial Ileal	0.13^{ab}	0.11^{b}	0.15^{a}	0.15^{a}	0.11^{b}	0.16^{a}	0.14^{ab}	0.02	0.02	0.10	0.65

1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.18 Average Crypt Depth (mm) of Intestinal Segments from Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Duodenum[2]	0.21	0.21	0.19	0.20	0.21	0.02	0.79
Medial Jejunum	0.14	0.17	0.17	0.17	0.15	0.01	0.48
Medial Ileum	0.13	0.14	0.15	0.16	0.14	0.01	0.31

1. Diets represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values reported as LSMEANS as calculated by proc MIXED

Table 4.19 Average Crypt Depth (mm) of Intestinal Segments from Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Duodenum[2]	0.22	0.19	0.22	0.19	0.20	0.20	0.21	0.02	0.80	0.16	0.95
Medial Jejunum	0.14	0.16	0.18	0.17	0.16	0.19	0.16	0.01	0.36	0.19	0.56
Medial Ileum	0.13	0.12	0.16	0.15	0.16	0.16	0.14	0.01	0.02	0.84	0.32

1 Diet: methionine: cystine ratio relative to requirement (100)
2 All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.20 Total Mucosa Depth (mm) for Intestinal Segments From Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Duodenum[2] (mm)	0.55	0.51	0.59	0.57	0.61	0.04	0.25
Medial Jejunum (mm)	0.43	0.50	0.48	0.48	0.44	0.03	0.09
Medial Ileum (mm)	0.42	0.47	0.51	0.53	0.51	0.03	0.56

1. Diets represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED

Table 4.21 Total Mucosa Depth (mm) for Intestinal Segments From Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100		P-value		
TSAA	50	75	75	100	150	150	200	Pooled SEM			
n	6	6	6	6	6	6	6		MET	CYS	CYS(ME
Duodenum[2,3] (mm)	0.55^{ab}	0.52^{ab}	0.50^{b}	0.59^{a}	0.51^{ab}	0.63^{a}	0.60^{a}	0.04	0.05	0.55	0.24
Medial Jejunum (mm)	0.44^{b}	0.42^{b}	0.55^{a}	0.49^{a}	0.47^{ab}	0.48^{ab}	0.44^{b}	0.03	0.01	0.39	0.33
Medial Ileum (mm)	0.42^{b}	0.43^{b}	0.52^{a}	0.50^{a}	0.50^{a}	0.55^{a}	0.51^{a}	0.03	0.004	0.75	0.84

1 Diet: methionine: cystine ratio relative to requirement (100)
2 All values reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3 Letter differences within a row indicates significance (P<0.05, Dunnetts) for MET, CYS and CYS(MET),

Table 4.22 Body Composition of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Crude Fat[2] (%)	9.32	8.60	8.19	7.98	8.45	0.63	0.45
Crude[2]Protein (%)	17.24	17.65	16.82	16.62	17.03	0.34	0.07
Moisture[3] (%)	67.04[b]	66.32[b]	68.71[a]	69.62a	68.98[a]	0.66	<0.0001
Ash (%)	4.37	4.89	4.36	4.91	4.71	0.35	0.54
Total	97.97	97.46	98.08	99.13	99.17	N/A	N/A
Protein: Fat	1.88	2.15	2.10	2.16	2.08	0.15	0.61

1. Diets represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED
3. Values reported as means with pooled SEM

Table 4.23 Body Composition of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Crude Fat[2,3] (%)	9.32[a]	7.73[b]	9.48[a]	8.19[ab]	8.13[ab]	7.82[ab]	8.45[ab]	0.67	0.91	0.03	0.83
Crude Protein (%)	17.24[b]	18.00[a]	17.29[b]	16.82[b]	16.16[b]	17.08[b]	17.03[b]	0.34	0.09	0.22	0.07
Moisture (%)	67.04[b]	66.68[b]	65.95[b]	68.71[a]	69.69[a]	69.56[a]	68.98[a]	0.73	0.20	0.01	0.01
Ash (%)	4.36	5.06	4.72	4.36	4.52	5.29	4.71	0.35	0.41	0.57	0.25
Total	97.99	97.42	97.45	98.24	98.49	99.75	99.16	N/A	N/A	N/A	N/A
Protein: Fat	1.88	2.37	1.92	2.10	2.08	2.23	2.08	0.15	0.75	0.06	0.54

1 Diet: methionine: cystine ratio relative to requirement (100)
2 All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within methionine intake
3 Letter differences within a row indicates significance ($P<0.05$, Dunnetts) for MET, CYS and CYS(MET)

Table 4.24 Plasma Amino Acids (mmol/L) for Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Indispensable							
Histidine[2,3]	0.06	0.08	0.06	0.07	0.05	0.01	0.65
Isoleucine	0.13	0.13	0.13	0.11	0.12	0.01	0.31
Leucine	0.25	0.42	0.26	0.20	0.22	0.14	0.61
Lysine	0.23	0.22	0.18	0.18	0.18	0.05	0.58
Methionine	0.04	0.02	0.03	0.03	0.03	0.007	0.42
Phenylalanine	0.07	0.11	0.04	0.06	0.06	0.04	0.61
Threonine	0.22	0.32	0.21	0.26	0.58	0.19	0.17
Tryptophan	0.04	0.04	0.04	0.06	0.03	0.02	0.56
Valine	0.30	0.29	0.27	0.28	0.28	0.02	0.71
Dispensable							
Alanine	0.62^{c}	1.07^{b}	1.25^{ab}	1.17^{b}	1.53^{a}	0.19	0.01
Arginine	0.10^{a}	0.12^{ab}	0.15^{b}	0.19^{b}	0.17^{b}	0.03	0.04
Aspartate	0.05	0.05	0.04	0.09	0.13	0.03	0.27
Cystine	0.03	0.05	0.05	0.05	0.03	0.02	0.22
Glutamate	0.24^{c}	0.27^{bc}	0.37^{b}	0.44^{ba}	0.55^{a}	0.08	0.02
Glutamine	0.60	0.58	0.59	0.54	0.56	0.05	0.78
Glycine	1.23^{a}	1.21^{a}	0.97^{a}	0.90^{ab}	0.86^{b}	0.13	0.003
Hydroxyproline	0.19	0.21	0.23	0.24	0.26	0.02	0.08
Proline	0.36	0.35	0.36	0.36	0.41	0.03	0.47
Serine	0.22	0.21	0.17	0.18	0.19	0.02	0.20
Taurine	0.04^{a}	0.07^{a}	0.07^{a}	0.23^{b}	0.26^{b}	0.03	<0.0001
Tyrosine	0.04	0.03	0.02	0.03	0.03	0.005	0.07

1. Diets represented as a percentage of NRC (1998) requirements, TSAA = total sulphur amino acid
2. All values reported as LSMEANS as calculated as proc MIXED
3. Letter differences within a row indicate significance ($P<0.05$, Dunnets)

Table 4.25 Plasma Amino Acids (mmol/L) for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Indispensable											
Histidine[2,3]	0.06^{bc}	0.05^{c}	0.12^{a}	0.06^{bc}	0.06^{bc}	0.08^{b}	0.06^{bc}	0.01	0.03	0.02	0.23
Isoleucine	0.13	0.13	0.14	0.13	0.12	0.10	0.12	0.01	0.37	0.38	0.50
Leucine	0.26	0.60	0.19	0.26	0.17	0.20	0.22	0.14	0.21	0.45	0.51
Lysine	0.23	0.23	0.21	0.20	0.17	0.17	0.16	0.05	0.59	0.28	0.32
Methionine	0.04	0.03	0.02	0.03	0.03	0.03	0.02	0.007	0.47	0.86	0.24
Phenylalanine	0.07	0.07	0.16	0.04	0.06	0.06	0.06	0.04	0.64	0.22	0.30
Threonine	0.22	0.34	0.29	0.20	0.30	0.21	0.49	0.19	0.58	0.28	0.32
Tryptophan	0.07	0.07	0.06	0.06	0.06	0.06	0.08	0.02	0.44	0.54	0.41
Valine	0.30	0.28	0.30	0.27	0.28	0.28	0.28	0.02	0.93	0.34	0.96
Dispensable											
Alanine	0.62^{b}	0.99^{ab}	1.20^{ab}	1.25^{ab}	1.12^{ab}	1.27^{ab}	1.53^{a}	0.19	0.05	0.62	0.26
Arginine	0.10	0.12	0.10	0.15	0.18	0.21	0.18	0.03	0.28	0.34	0.49
Aspartate	0.05	0.07	0.03	0.04	0.08	0.09	0.13	0.03	0.42	0.32	0.98
Cystine	0.02	0.04	0.06	0.06	0.04	0.07	0.03	0.02	0.06	0.35	0.46
Glutamate	0.24	0.26	0.29	0.37	0.41	0.48	0.56	0.08	0.09	0.43	0.93
Glutamine	0.60	0.54	0.61	0.60	0.51	0.56	0.56	0.05	0.70	0.21	0.75
Glycine	1.23^{a}	1.16^{ab}	1.32^{a}	0.97^{ab}	0.90^{b}	0.89^{b}	0.80^{b}	0.13	0.51	0.01	0.40
Hydroxyproline	0.19^{b}	0.19^{b}	0.24^{a}	0.23^{ab}	0.24^{a}	0.24^{a}	0.25^{a}	0.02	0.02	0.86	0.93
Proline	0.36	0.36	0.35	0.36	0.36	0.37	0.41	0.03	0.39	0.76	0.77
Serine	0.22^{a}	0.23^{a}	0.19^{ab}	0.17^{b}	0.18^{ab}	0.19^{ab}	0.18^{ab}	0.02	0.03	0.94	0.88
Taurine	0.04^{b}	0.06^{b}	0.07^{b}	0.08^{b}	0.25^{a}	0.21^{a}	0.26^{a}	0.03	0.02	0.003	0.24
Tyrosine	0.04	0.03	0.03	0.02	0.03	0.02	0.03	0.005	0.06	0.24	0.88

1 Diet: methionine: cystine ratio relative to requirement (100)

2 Letter differences within a row indicates significance ($P<0.05$, Dunnetts) for MET, CYS and CYS(MET)

3 All values reported as LSMEANS as calculated by proc MIXED, , MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.26 Liver Amino Acids (μmol/g wet weight) For Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 Days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Indispensable							
Histidine[2]	0.55	0.70	0.57	0.55	0.84	0.17	0.59
Isoleucine	0.94	2.00	1.62	1.73	1.62	0.48	0.38
Leucine	1.52	2.22	2.05	2.20	2.21	0.38	0.55
Lysine	1.35	1.39	1.34	1.42	2.09	0.48	0.72
Methionine	0.59	1.03	0.90	1.14	1.20	0.19	0.09
Phenylalanine	0.56	0.79	0.76	0.87	1.20	0.17	0.09
Threonine	3.65	2.02	2.57	1.43	2.17	0.65	0.10
Tryptophan	0.67	1.16	1.08	1.28	1.31	0.16	0.35
Valine	1.13	1.64	1.62	1.72	1.79	0.27	0.37
Dispensable							
Alanine	5.67	6.73	8.49	6.73	8.99	1.22	0.11
Arginine	0.15	0.24	0.30	0.28	0.33	0.05	0.09
Aspartate	2.29	3.32	3.42	3.21	3.12	0.82	0.60
Cystine	0.19	0.27	0.50	0.55	0.79	0.20	0.15
Glutamate	4.40	5.10	3.64	4.41	2.25	1.54	0.65
Glutamine	2.25	2.79	4.32	4.75	4.72	1.14	0.25
Glycine	11.81	12.66	13.88	14.23	11.06	1.67	0.38
Hydroxyproline	0.47	0.55	0.69	0.61	0.69	0.16	0.81
Proline[3]	1.29^{c}	2.56^{b}	1.65^{b}	3.70^{a}	4.95^{a}	0.52	<0.0001
Serine	5.42^{a}	3.90^{b}	3.11^{b}	3.00^{b}	4.28^{ab}	0.83	0.03
Taurine	5.39^{a}	7.58^{a}	7.81^{a}	15.14^{b}	16.45^{b}	3.32	0.04
Tyrosine	0.79	1.03	0.99	0.98	1.16	0.16	0.59

1. Diets are represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED
3. Letter differences within a row ($P < 0.05$, Dunnetts) indicates signficance

Table 4.27 Liver Amino Acids (μmol/g wet weight) For Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 Days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Indispensable											
Histidine2	0.55	0.59	0.83	0.57	0.55	0.55	0.84	0.17	0.82	0.84	0.42
Isoleucine	0.94	1.69	2.38	1.62	1.66	1.80	1.62	0.48	0.22	0.70	0.16
Leucine	1.52	1.72	2.83	2.05	2.29	2.11	2.21	0.38	0.21	0.92	0.35
Lysine	1.35	1.41	1.36	1.34	1.45	1.38	2.10	0.48	0.68	0.59	0.81
Methionine	0.59	1.01	1.05	0.90	0.99	1.29	1.20	0.19	0.25	0.37	0.36
Phenylalanine	0.56	0.68	0.93	0.76	0.88	0.87	1.20	0.17	0.35	0.49	0.49
Threonine	3.65	2.45	1.51	2.57	1.70	1.16	2.17	0.65	0.05	0.82	0.10
Tryptophan	0.79	0.84	1.26	0.99	1.01	0.95	1.16	0.16	0.40	0.86	0.40
Valine	1.13	1.53	1.77	1.62	1.71	1.74	1.79	0.27	0.72	0.59	0.65
Dispensable											
Alanine	5.67	5.89	7.76	8.49	7.25	6.21	8.99	1.22	0.50	0.60	0.15
Arginine[3]	0.15^{a}	0.17^{a}	0.34^{c}	0.30^{bc}	0.27^{b}	0.29^{bc}	0.33^{bc}	0.05	0.005	0.86	0.41
Aspartate	2.29	3.50	3.10	3.42	3.31	3.42	3.12	0.82	0.66	0.63	0.73
Cystine	0.19	0.20	0.35	0.50	0.66	0.44	0.79	0.20	0.38	0.36	0.88
Glutamate	4.40	6.48	3.41	3.64	5.04	3.79	2.25	1.54	0.31	0.72	0.63
Glutamine	2.25	2.34	3.33	4.32	5.73	3.77	4.72	1.14	0.30	0.41	0.93
Glycine	11.81^{ab}	10.72^{b}	15.03^{a}	13.88^{a}	14.38^{a}	14.08^{a}	11.06^{ab}	1.67	0.02	0.37	0.44
Hydroxyproline	0.47	0.45	0.67	0.69	0.48	0.74	0.69	0.16	0.13	0.53	0.86
Proline	1.29^{c}	2.36^{b}	2.81^{b}	1.65^{bc}	4.16^{a}	3.23^{b}	4.95^{a}	0.52	0.10	0.003	0.12
Serine	5.42	4.07	3.69	3.11	2.93	3.07	4.28	0.83	0.10	0.14	0.51
Taurine	5.39^{b}	10.00^{b}	4.59^{b}	7.81^{b}	18.41^{a}	11.41^{ab}	16.45^{a}	3.32	0.93	0.02	0.65
Tyrosine	0.79	0.84	1.26	0.99	1.01	0.95	1.16	0.16	0.40	0.86	0.40

1. Diet: methionine: cystine ratio relative to requirement (100)
2. Letter differences within a row indicates significance ($P<0.05$, Dunnetts) for MET, CYS and CYS(MET)
3. Means are reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.28 Kidney Free Amino Acids (µmol/g wet weight) of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Indispensable							
Histidine[2]	0.21	0.30	0.28	0.21	0.17	0.06	0.15
Isoleucine	0.55[ab]	0.71[a]	0.70[a]	0.61[ab]	050[b]	0.08	0.05
Leucine	1.18	1.44	1.44	1.28	1.14	0.18	0.21
Lysine[3]	0.60[b]	0.65[b]	0.61[b]	0.90[a]	0.82[a]	0.09	0.04
Methionine	0.64[b]	0.63[b]	0.25[b]	0.47[ab]	0.56[b]	0.15	<0.0001
Phenylalanine	0.45	0.56	0.57	0.47	0.43	0.06	0.22
Threonine	0.61	0.61	0.38	0.65	0.89	0.15	0.17
Tryptophan	0.06	0.28	0.08	0.11	0.24	0.11	0.28
Valine	1.06	1.20	1.22	1.13	1.00	0.13	0.60
Dispensable							
Alanine[3]	1.89[a]	2.07[a]	1.50[a]	1.67[a]	3.96[b]	0.72	0.03
Arginine[3]	0.38	0.46	0.47	0.41	0.39	0.06	0.68
Aspartate	0.17	0.20	0.66	0.20	0.16	0.17	0.16
Cystine	0.28	0.40	0.11	0.45	0.34	0.12	0.11
Glutamate	2.10	2.06	3.41	2.32	2.26	0.45	0.13
Glutamine[3]	1.06	1.18	1.45	1.17	1.13	0.11	0.15
Glycine	7.76	8.47	7.63	6.83	7.50	0.67	0.24
Hydroxyproline	0.58	1.01	0.62	0.67	0.80	0.16	0.19
Proline	1.58	1.82	1.61	1.69	1.66	0.22	0.81
Serine	1.69	1.71	1.83	1.74	1.55	0.24	0.93
Taurine[3]	3.70[a]	3.93[ab]	4.81[b]	6.71[c]	6.34[c]	0.61	<0.0001
Tyrosine	0.56	0.63	0.65	0.58	0.53	0.74	0.68

1. Diets are represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid
2. All values reported as LSMEANS as calculated by proc MIXED
3. Letter differences within a row indicates significance (P<0.05, Dunnets)

Table 4.29 Kidney Free Amino Acids (μmol/g wet weight) of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Indispensable											
Histidine[2]	0.21	0.23	0.28	0.28	0.22	0.21	0.17	0.06	0.26	0.31	0.33
Isoleucine	0.55	0.67	0.75	0.69	0.59	0.63	0.50	0.08	0.10	0.08	0.42
Leucine	1.18	1.34	1.51	1.44	1.25	1.31	1.14	0.18	0.19	0.23	0.62
Lysine[3]	0.60	0.59	0.71	0.61	0.82	0.99	0.82	0.09	0.04	0.97	0.16
Methionine	0.64	0.53	0.71	0.25	0.30	0.60	0.56	0.15	0.21	0.10	0.51
Phenylalanine	0.45	0.52	0.59	0.57	0.42	0.52	0.43	0.06	0.35	0.06	0.55
Threonine	0.61	0.46	0.73	0.38	0.61	0.68	0.89	0.15	0.14	0.13	0.79
Tryptophan	0.06	0.14	0.39	0.08	0.15	0.07	0.24	0.11	0.53	0.34	0.10
Valine	1.06	0.99	1.36	1.22	1.08	1.18	0.99	0.13	0.09	0.14	0.95
Dispensable											
Alanine	1.89^{ab}	1.29^{b}	2.72^{ab}	1.50^{ab}	1.59^{ab}	1.75^{ab}	3.93^{a}	0.72	0.08	0.06	0.12
Arginine	0.38^{ab}	0.35^{b}	0.58^{a}	0.47^{a}	0.41^{ab}	041^{ab}	0.39^{ab}	0.06	0.04	0.20	0.83
Aspartate	0.17	0.16	0.23	0.67	0.18	0.22	0.16	0.17	0.29	0.34	0.30
Cystine	0.28	0.38	0.42	0.11	0.39	0.51	0.34	0.12	0.41	0.87	0.05
Glutamate	2.10	2.03	2.09	3.41	2.25	2.38	2.26	0.45	0.34	0.35	0.22
Glutamine	1.06^{b}	1.00^{b}	1.32^{ab}	1.45^{a}	1.09^{b}	1.24^{ab}	1.13^{b}	0.11	0.01	0.11	0.43
Glycine	7.76	7.58	9.21	7.63	6.74	6.91	7.49	0.67	0.73	0.13	0.23
Hydroxyproline	0.58	0.71	1.26	0.62	0.68	0.67	0.80	0.16	0.26	0.16	0.06
Proline	1.56	1.61	1.99	1.61	1.61	1.77	1.66	0.22	0.44	0.42	0.53
Serine	1.68	1.43	1.94	1.83	1.64	1.83	1.55	0.24	0.29	0.35	0.72
Taurine	3.40^{c}	4.39^{bc}	3.54^{bc}	4.81^{b}	6.90^{a}	6.52^{a}	6.35^{a}	0.61	0.21	0.003	0.09
Tyrosine	0.56	0.53	0.71	0.65	0.56	0.61	0.53	0.74	0.16	0.21	0.98

1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3. Letter differences within a row indicates significance ($P<0.05$, Dunnets) for MET, CYS and CYS(MET)

Table 4.30 Medial Jejunal Protein Content (mg Protein/ g Tissue) for Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Mucosa Protein[2]	14.91	13.68	15.04	14.80	13.07	2.50	0.92

1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.31 Medial Jejunal Protein Content (mg Protein/ g Tissue) for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Mucosa Protein[2]	14.91	12.16	15.19	15.04	13.09	16.54	13.04	2.50	0.50	0.37	0.76

1. 1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values reported as LSMEANS as calculated by proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

Table 4.32 Enzyme Profile (Units/g of jejunum) for Piglets Consuming Experimental Diets Containing Various Concentrations of Sulphur Amino Acids for 7 days

Diets[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	5	12	6	12	5		
Lactase[2]	51.85	38.24	36.89	49.49	47.97	15.75	0.76
Maltase	71.58	133.29	128.72	90.34	134.58	38.94	0.56
Sucrase	44.42	46.48	62.99	30.14	36.73	21.16	0.51

1. Diets are represented as a percentage of NRC (1998) requirement, TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED

Table 4.33 Enzyme Profile (Units/g of jejunum) for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of Sulphur Amino Acids for 7 days

Diets[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled SEM	P-value		
TSAA	50	75	75	100	150	150	200		MET	CYS	CYS(MET)
n	5	6	6	6	5	6	6				
Lactase[2]	51.85	37.49	39.14	36.89	46.40	54.21	47.47	15.75	0.69	0.87	0.68
Maltase	71.58	181.80	84.58	128.72	111.55	68.89	132.58	38.94	0.35	0.16	0.29
Sucrase	44.30	41.25	53.05	55.71	29.43	45.40	36.73	21.16	0.78	0.20	0.62

1 Diet: methionine: cystine ratio relative to requirement (100)

2 All values expressed as LSMEANS as calculated as proc MIXED, MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake

requirements are based on extrapolations of growth curves for the lysine requirements of heavier, and thereby older pigs. Using the ideal protein model for protein accretion, coupled with the lysine requirement, the requirements for other amino acids were estimated. Without sufficient empirical evidence to support these recommendations it is unclear whether piglets are receiving sufficient amino acids, such as MET and CYS_2, to support protein accretion and other biological functions required to achieve optimal growth. NRC (1998) recommends that the total sulphur amino acid (TSAA) requirement of 3-5 kg piglets is 0.50 g kg^{-1} d^{-1}), however recent research in our laboratory utilizing the indicator amino acid method determined the requirement for methionine to be 0.42 g kg^{-1} d^{1} for this weight class of pig (Shoveller et al., 2003a). As MET can meet 100% of the TSAA requirement, it is apparent from the aforementioned data that the requirement for TSAA is lower than that recommended in NRC (1998).

The first objective of this trial was to examine the effect of increasing concentration of TSAA on the growth and gastrointestinal development of the SEW piglet. Utilizing diets that ranged in TSAA concentration (from 50% to 200% of NRC) provided the necessary range to study the effect of various concentrations of MET and CYS_2 on the overall development of 3-5 kg piglets (Chung and Baker, 1992a). These diets were formulated to avoid any metabolic disorders in the piglets while still providing a great enough difference across possible TSAA intakes to measure physiological differences.

The second objective of this trial was to examine the effect of ratio of MET to CYS_2 on the growth and gastrointestinal development of SEW piglets. While MET can meet 100% of the TSAA requirement it has long been known that CYS_2 can spare MET (Becker et al., 1954, Kroening et al., 1965, Chung and Baker, 1992b). NRC (1998) recommends that CYS_2 make up 50% of the TSAA but the experimental values ranged from 40-70%. However, when Chung and Baker (1992b) fed piglets diets containing 40% MET and 60% CYS_2 they observed decreased rates of growth as compared to piglets receiving diets with 50-50% or 60-40% MET: CYS_2. To the best of the authors' knowledge Chung and Baker (1992b) is the only previous published research that included diets where CYS intake exceeded MET intake. Also, further work in our laboratory has determined, utilizing the indirect amino acid oxidation method, that the maximum sparing capacity of cysteine (CYS) on the MET requirement is less than 50% of the TSAA requirement (Shoveller et al., 2003b). Utilizing diets that were 1:1, 1:2 or 2:1 (MET:CYS_2) provided the necessary comparisons to examine physiological differences.

The growth results from this trial illustrate diets deficient not only in TSAA (i.e. diet 25-25; 50% of TSAA requirement) but also of MET (diets 25-25 and 25-50, 25% of MET requirement) decreased gain, feed intake and feed efficiencies. As the level of MET inclusion in the diets increased to 50% of the TSAA requirement (0.25 g kg^{-1} d^{-1}) so too did gain, intake and efficiency (Table 4.2, Figure 4.1). However, when the TSAA concentration of the diet increased (150% and 200% of NRC) and MET inclusion increased to 100% (0.50 g kg^{-1} d^{-1} of methionine, diets 100-50 and 100-100)

resulted in lower gain, intake and feed efficiency, diet 50-25 results were surprising. This diet was formulated to be 75% of the NRC requirement (0.375 g kg^{-1} d^{-1}), yet piglets consuming this diet performed identically to those receiving diet 50-50 (0.50 g $kg^{-1}d^{-1}$) that met the NRC recommended requirements for the TSAA. These results indicate that although the requirement for MET to be 50% of the TSAA requirement is most likely correct, the requirement for TSAA inclusion in the diet to be 0.50 g $kg^{-1}d^{-1}$ for SEW piglets of 3-5 kg may be less than the current recommendation. Similar growth rates, feed intakes and feed efficiencies in response to MET intake have been reported by other researchers investigating MET requirements in 3-5 kg piglets as well as in older and heavier pigs (Leibholz et al., 1984, Chung and Baker, 1992a,b and Owen et al., 1995).

As the CYS_2 intake within a MET group (i.e. 25%, 50% and 100%) increased ADG, ADFI and G: F decreased. Piglets consuming diet 25-25 had greater ADG, ADFI and better G: F efficiencies than those consuming diet 25-50. One would assume that the increased dietary CYS_2 would have acted to spare the dietary MET for use in growth and other biological functions. While diet 50-25 was deficient according to the TSAA requirement (NRC, 1998) the piglets consuming this diet grew identically to those consuming diet 50-50. As the concentration of the TSAA content of the diet increased to 150% and the dietary CYS_2 increased there was a decrease in ADG and ADFI, it is apparent that there is a pattern of increasing CYS_2 resulting in decreased gain. These results were supported by similar observations made by Chung and Baker (1992b) where piglets fed a diet with a MET intake (40% of TSAA

requirement) less than that of CYS_2 (60% of TSAA requirement) had lower growth rates as compared to those piglets consuming diets in which MET made up 50 or 60% of the TSAA requirement. However, it appears that increased CYS_2 acted to inhibit growth. A similar observation was made for piglets consuming diets 100-50 and 100-100 (Table 4.2). As the intake of dietary CYS_2 increased, subsequent decreases in gain, intake and feed efficiency were observed. These results indicate that increasing dietary CYS_2 beyond 50% of MET inclusion has detrimental effects on the growth of piglets.

A lack of increasing nitrogen retention as TSAA increased beyond 75% (0.375 g $kg^{-1}d^{-1}$) of NRC further supports the conclusion that the current NRC (1998) recommendation of 0.50 g $kg^{-1}d^{-1}$, (Table 4.3) may be too high. As dietary MET was increased to 50% (0.25 g $kg^{-1}d^{-1}$) of the TSAA requirement (50-25) as opposed to 25% in diets 25-25 and 25-50 and TSAA concentration was increased to 75% (0.375 g $kg^{-1}d^{-1}$), nitrogen retention and therefore protein accretion increased. Further increases of TSAA beyond 75% did not increase nitrogen retention, and increasing the MET concentration to 100% of the TSAA requirement resulted in lower gains, feed intakes, feed efficiencies and nitrogen retention.

Plasma urea nitrogen values are used as an indicator of urea cycle function. The more urea nitrogen that is in the plasma, the more nitrogen is lost, that is, unavailable for protein synthesis. As the TSAA content of the diets increased, plasma urea nitrogen values decreased indicating an increased utilization of circulating nitrogen. Plasma

urea nitrogen values decreased as dietary MET increased to 50% of the TSAA requirement and then remained constant (Table 4.3). For diets 25-25 and 25-50, piglet plasma urea concentrations on day 6 ($P < 0.0001$, Table 4.4) were greater than those of piglets consuming adequate and excess MET indicating increased nitrogen in the urea cycle. On day 10, a similar effect was observed, as dietary MET intake increased to 50% of the TSAA requirement, the amount of plasma nitrogen decreased. These results coupled with lower ADG, ADFI and gain to feed efficiencies indicate that piglets receiving inadequate methionine were less able to convert dietary nitrogen into protein that the pigs that received adequate TSAA and MET intakes.

These present results are often seen during the feeding of diets with amino acid imbalances (Harper et al., 1970). As the pigs consume less feed containing a severely disproportionate ratio of MET to CYS_2 (i.e. 50-100) they would retain less nitrogen and thereby gain less weight and result in decreased thriftiness. Another possible explanation for the resulting decreases in gain, intake and feed efficiencies could be the increased production of sulphate resulting from CYS oxidation. However, as TSAA dietary concentration increased, so too did the plasma taurine concentration ($P < 0.0001$, Table 4.13). Increasing plasma taurine concentration indicates that the pigs may not have reached the limit of taurine synthesis to sequester sulphate; Stipanuk et al., (1986) reported that in rat enterocytes as much as 80% of CYS oxidised resulted in taurine synthesis. The same mechanism may be present in pigs, and as a consequence, an increase in plasma sulphate level seems unlikely.

On day 10 of the trial piglets were killed and samples were removed for further study. The weight of the liver and kidney was affected by TSAA intake. As TSAA intake increased so too did the weight of the liver and kidney. Piglets consuming diets 25-25 and 25-50 had lighter livers than those piglets consuming diets 50-25, 50-50 and 50-100. However, for piglets consuming diet 100-50 and 100-100 a decrease in the weight of the liver occurred. As the TSAA concentration of the diet increased the weight of the kidney increased. There was a trend for increasing dietary MET intake to increase the weight of the spleen ($P = 0.09$, Table 4.5). Also, as dietary CYS_2 increased so too did the weight of the kidney, curiously in opposition to other datasets, kidney weights increased with increased dietary CYS_2. As dietary CYS_2 increased so too did the taurine concentration in the kidney ($P = 0.003$, Table 4.15). Taurine plays a large role in maintaining osmoregularity in the cell (Schaffer et al., 2000). Increased taurine concentration intra-cellularly may have lead to cellular hypertrophy and thus heavier kidneys.

Increasing MET intake affected small intestinal weight and length ($P = 0.02$, $P = 0.01$ respectively, Table 4.6). MET deficient diets 25-25 and 25-50 did not support similar growth, ADG, ADFI or nitrogen retention as those diets that contained adequate MET concentrations (50-25, 50-50, 50-100, 100-50 and 100-100). Although not significant, increasing dietary CYS_2 intake within a MET group negatively affected the length of the small intestine. Specific sections of the small intestine: the duodenum, proximal and medial jejunum and ileum were excised for analysis.

Increasing dietary MET intake increased all measured parameters for the jejunum: jejunal weight, jejunal mucosal weight and mucosal weight per length (P = 0.02 for all parameters, Tables 4.7, 4.8, 4.9). Increasing dietary CYS_2 concentrations negatively affected the medial jejunum for all parameters measured, jejunal weight, and mucosal weight and for (g) mucosa per cm of medial jejunum (P = 0.10, Tables 4.7, 4.8 and 4.9). The jejunum is the site of most amino acid absorption and metabolism as such the decreases observed indicate that dietary CYS_2 must be negatively impacting intestinal metabolism. In the medial ileum MET intake reduced mucosal mass (P = 0.01, Table 4.8) and there was a trend (P = 0.07, Table 4.9) for increasing dietary CYS_2 intake to reduce the mucosal mass per cm of ileum. Increased dietary CYS_2 intake negatively acts throughout the small intestine. Why this occurs is unclear however, we speculate that a metabolite of CYS_2 may have a negative impact on the gut when included in the diet at concentrations greater than 50% of MET inclusion in the diet. In the medial jejunum, increasing dietary CYS_2 intake decreased the villus height and the depth of the mucosa. This was also observed in the medial ileum for the total depth of the mucosa. This difference was the result of TSAA -deficient versus adequate diets. A possible explanation of the reduction in villus height and crypt depth may be that increased dietary CYS_2 exerts a mucolytic effect, thereby altering the mucus structure and function of the small intestine via increased glutathione production. Precursors of cysteine, and thereby glutathione, such as N-acetylcysteine are used to breakdown the mucus of patients suffering from conditions such as cystic fibrosis (Hudson, 2001). Excess dietary CYS_2 may have had a similar effect on the mucosa of the small intestine. The result

of this mucolytic effect may be that, a reduction in the mucus layer protecting the gut acted to reduce villus height and crypt depth via direct exposure to the lumenal contents of the gut. And, that this decrease in depth and height reduces the absorptive area and decreases the enterocytic maturity as it migrates to the tip of the villus. The decreased absorptive area reduces total absorption and decreased enterocyte maturity decreases the absorptive capacity of the gut. Further, we speculate the loss of the protective mucus layer may result in localized inflammation exacerbating the decreased feed intake observed by increasing cytokine production that acts to inhibit or reduce feed intake similar to that observed over the course of this trial.

There did not appear to be any effect of MET or CYS_2 intake upon the protein content nor the enzyme profile of the medial jejunum (Table 4.16). There was however, a non-significant pattern of increasing dietary CYS_2 decreasing the protein content of the small intestine. Also, there was no effect of MET or CYS_2 intake on the enzymatic profile of the jejunum (Table 4.17). However, as dietary CYS_2 intake increased there was an increase in the maltase concentration.

4.18 Conclusions

Based on the results of this trial, it is evident that increasing the TSAA percentage above 75% of the current NRC (1998) recommendations provides no benefits to SEW piglets. Further, this data supports the findings of Shoveller et al., (2003a) in which the requirement was determined to be 84% (0.42 g $kg^{-1}d^{-1}$) of the NRC recommendation.

It appears from the reoccurring pattern of increased dietary CYS_2 negatively affecting numerous measurements in this trial that the relationship between MET and CYS_2 is a delicate balance. Current recommendations for equal intakes of MET and CYS_2 may be overstated as inclusion of CYS_2 in diets beyond a 2:1 ratio of MET to CYS_2 resulted in decreased growth and gastrointestinal development in SEW piglets. These results support the findings of Shoveller et al., (2003b) that the relationship of MET to CYS is 60: 40.

5.0 Rationale and Objectives

5.1 Rationale

In Experiment 1, the affects of feeding different ratios and concentrations of MET and cystine (CYS_2), for 7 days on growth and gut development in early-weaned piglets was examined. The inclusion of dietary CYS_2 above 50% of methionine inclusion had a consistently negative although sometimes as a trend ($P<0.1$), impact on the growth and gastrointestinal structure of the early-weaned piglet. Increasing dietary CYS_2 concentration negatively affected gain, feed intake and gross and histological gastrointestinal parameters. In the small intestine, increasing dietary CYS_2 significantly decreased medial jejunal mucosa weight and the total depth of the medial jejunal and medial ileal mucosa. From this experiment we concluded that CYS_2 has a role in gut metabolism and development however, the nature of this role is unknown.

N-acetylcysteine (NAC) is commonly used as an antioxidant and mucolytic agent. NAC is a precursor to cysteine (CYS) and thereby, to glutathione (GSH) (De Vries and De Flora, 1993). CYS is converted to GSH via 2 enzymatic steps. First, CYS and glutamate are converted to γ-Glutamylcysteine via γ-glutamylcysteine synthetase (EC 6.3.2.2). The second enzyme in the procedure, glutathione synthetase (EC), adds glycine to form the tripeptide glutathione. GSH is a ubiquitous molecule found in every tissue of the body and is one of the primary compounds responsible for maintaining redox status in the cell. Glutathione also acts as a mucolytic agent. Glutathione –SH group hydrolyses the disulfide bonds attached to CYS residues of

mucus. In patients with diseases such as cystic fibrosis, NAC is used to increase the fluidity of mucus to aid in the expectoration of pulmonary mucus. Because NAC is an effective precursor to CYS in the neonatal piglet (Shoveller, Ph.D Thesis 2004), its mucolytic effects are likely exerted by its conversion to CYS and then incorporation into glutathione.

In the gastrointestinal tract, the mucus layer protects the delicate tissues and structures of the gut. The function of the mucus is to trap pathogens and protect the gut from physical damage (Vente-Spreeuwenberg and Beynen, 2003). In the gut, GSH sources are: diet, secretion by the mucosa and from the liver via the biliary duct (Dahm and Jones, 1994). In the small intestine under normal conditions, GSH increases mucus fluidity and acts as a scavenger of free radicals and toxic compounds that may be ingested with feed (Loguercio and Di Pierro, 1999). However, in the gastrointestinal tract increased fluidity of the protective mucus layer may be detrimental. CYS is important for mineral absorption across the lumen of the gut (Wein and Van Campen, 1991) and GSH plays a vital role in protection of the small intestine from oxidant attack. However, excesses of either molecule, from dietary sources or endogenous synthesis, may interfere with the normal function of the piglet small intestine. An increase in fluidity and a decrease in the depth of the mucosa, may increase the risk of exposure of the tissue surface to lumenal contents and thereby increase the risk of damage or pathogenic bacterial translocation.

In Experiment 1 (Supplementation of Sulphur Amino Acids in Early-Weaned Piglets) it was observed that increased dietary CYS_2 inhibited growth and

negatively affected gastrointestinal structure. We speculated that the increasing dietary CYS_2 lead to an overproduction of GSH that acted as a mucolytic agent in the small intestine. The excess GSH may have increased the fluidity of the mucus lining the small intestine, resulting in the observed thinning of the mucosa depth. Currently, it is known that increased dietary CYS increases plasma GSH concentrations (Badaloo et al., 2002) but it is unclear if a similar response of increasing dietary CYS increases mucosal GSH concentration occurs.

In order to examine if increased dietary CYS increases mucosal GSH concentration, a range of dietary cysteine concentrations was utilized along with a means of measuring the formation of GSH from the dietary CYS. Infusion of L-[1-^{14}C] Cysteine allowed the determination of the incorporation of dietary CYS into GSH. Using a wide range of dietary intakes of CYS would permit an examination of the relationship between dietary cysteine and GSH concentration in early weaned piglets receiving an elemental diet.

5.2 Hypotheses

In order to examine the effect of increasing CYS intake on glutathione synthesis the following hypotheses were devised.

1. The incorporation rate of CYS into mucosal, hepatic and renal total glutathione will increase when dietary CYS intake increases, and methionine intake is held constant.

2. There will be an increase in the ratio of 2GSH: Glutathione disulfide (GSSG) when the dietary CYS intake increases and MET intake is held constant.

5.3 Objectives

In order to test the validity of the hypotheses the following objectives were devised:

1. To measure the effect of dietary CYS intakes (0.0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.40, and 0.50 g/kg/d) on the incorporation of CYS into total glutathione in the mucosa, liver and kidney using L-[1-^{14}C] Cysteine infusion.
2. To examine the ratio of 2GSH: GSSG, an indicator of cellular redox status, in the mucosa, liver and kidney of piglets receiving graded intakes (0.0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.40, and 0.50 g/kg/d) of dietary CYS.

6.0 L [1-^{14}C]-Cysteine Supplementation and Glutathione Synthesis

6.1 Materials and Methods

The Faculty Animal Policy and Welfare Committee at the University of Alberta approved all surgical, daily care and animal sampling procedures prior to the start of this experiment.

Thirty-two, 2-day old male, intact piglets weighing 1.3-1.8 kg were selected for the experiment. Piglets were housed in circular mesh cages equipped with a swivel-tether system (Alice King Chatham Medical Arts, Los Angeles, CA. USA) and allowed visual and auditory interaction. Supplemental heat was provided via a heat-lamp to maintain ambient temperature at piglet level at approximately 25°C. Piglets were provided with toys for environmental enrichment.

6.1.1 Surgery

Piglets were initially anaesthetised using an Isofluorane® and oxygen mixture at a flow rate of 5 ml/min. Piglets were intubated and anaesthesia maintained using a flow rate of 4 ml/min. Piglet respiration rate, heart rate and blood oxygen status were monitored throughout the surgery.

A silicastic catheter was inserted into the femoral vein and was advanced to the inferior vena cava. This catheter was used for blood sampling. A silicastic gastric catheter was installed using the technique described by Rombeau (1984). The gastric catheter was used for diet and isotope infusion.

Post-operatively, piglets were fitted with an adjustable cotton jacket and attached to the swivel-tether system within their cage. The swivel-tether system prevents catheter tangling and occlusion during constant dietary infusion and allows piglet freedom of movement within their cages. Each piglet was continuously monitored until it had regained consciousness. Piglets received 0.5 ml sulfadoxine (Borgal, Hoechst Roussel Vet Canada Inc, Regina, SK. Canada) and 0.2 ml Buprenex® (Buprenex, Reckitt and Colman Pharmaceuticals Inc, Richmond, VA, USA) post-operatively. However, piglets did not receive Buprenex® until they had regained consciousness from surgery.

Intravenous feeding of the elemental diet occurred for the first 8-12 hours post operatively at 50% (~ 6.75 ml kg^{-1} d^{-1}) of the target rate of ~15 ml kg^{-1} d^{-1}. After 8-12 hours the rate was adjusted to 75% (~10.75 ml kg^{-1} d^{-1}). At 24 hrs post surgery the route of feeding was altered to intra-gastric feeding. Once again the pump rate was scaled up from 50% to 100% (13.5 ml kg^{-1} d^{-1}) for the elemental diet over a 24-hour period for the intra-gastric infusion of diet.

6.1.2 Daily Care

Piglets were maintained on a 12 h light: dark schedule. Every morning at 07:00 h the lights were turned on, piglets were inspected for functioning catheters, and general well-being while pumps were inspected for occlusions. Each animal was weighed and all incisions were treated with Hibitane® (Ayerst Laboratories, Montreal, PQ, Canada) to prevent infection.

Diet bags were weighed and differences between previous weights were used to calculate pump efficiencies. The rate of diet infusion (13.5 ml kg^{-1} d^{-1}) was then corrected for piglet weight and for pump infusion efficiency. Throughout the day piglets were observed for health and well-being. Daily at 19:00 h piglets were inspected for jacket security, tether tightness; pump efficiency and well-being before the lights were turned off. At midnight, a final check occurred to ensure piglet health until 07:00 h the following morning.

6.1.3 Diet

Complete diets were formulated to supply all nutrients necessary for growth. Diets were mixed in batches of 20 L from which 19 units of 750 ml was filtered (0.22 μm Millipore Corporation, Bedford, MA. USA) and pumped into sterile infusion bags. The diet was stored in the dark at 4°C and used within 5 days.

The amino acid profile of complete diet was patterned after that of Vaminolact (Fresenius-Kabi Nutrition, Germany), commonly used in infant neonatal total parenteral nutrition, with the following exceptions: arginine was provided at 1.2 $g/kg^{BW}/d$ (Brunton et al, 1999) and phenylalanine and tyrosine which were provided at their safe levels of intake (House, 1997a, House et al, 1997b). Target intakes were, 15 $g/kg^{BW}/d$ amino acids, 25$g/kg^{BW}/d$ glucose, 11$g/kg^{BW}/d$ lipid and a metabolisible energy of 1.1 $MJ/kg^{BW}/d$ for the complete diet. Vitamins (Multi-12K_1 Pediatric, Bouchervill, PQ. Canada), minerals and lipid (Intralipid 20%, Pharmacia Inc., Mississauga, ON. Canada) were added to diet bags immediately prior to infusion.

Minerals were administered at 200% the NRC (1998) recommendations to ensure meeting requirement.

Test diets were formulated identically to the complete diet except that, MET was held constant at 0.25 g kg^{-1} d^{-1} (50% TSAA requirement, NRC 1998) while CYS concentrations, provided as CYS-free base, ranged from 0, 0.05, 0.1, 0.15, 0.2, 0.25, 0.40 and 0.5 g kg^{-1} d^{-1}. MET concentration was held constant in order to investigate the sole effects of increasing dietary CYS. The range of dietary CYS permitted investigation of a dose-effect of increasing dietary CYS on GSH concentration. Diets were kept isonitrogenous utilizing alanine to balance nitrogen intake.

Piglets were maintained on complete diet from surgery until the day prior to oxidation (day 0 – 5). 16 h prior to oxidation, piglets were randomly assigned to one of the eight test diets. Piglets were maintained on test diet for 3 days until the completion of the oxidation on day 8.

6.1.4 Oxidation: Day 6 – Day 8

On the morning of day 6, all piglets were placed in a Plexiglas® metabolic chamber and underwent a 6 h primed, constant infusion isotope tracer experiment. Each piglet received a priming dose (5 μCi/kg) of L-[1- ^{14}C]CYS (American Radiolabeled Chemicals, Inc. St. Louis, MO. USA). After receipt of the priming dose, piglets were constantly infused (5 μCi/kg/h) for 6 hrs. Expired $^{14}CO_2$ was collected quantitatively in gas washing bottles containing liquid carbon dioxide absorber (Results not

included in this thesis, please see Shoveller, 2004) (2-methoxyethanol : ethanolamine, 2:1). During the oxidation, blood samples were taken every half hour (see description below). After the 6 h oxidation, piglets were returned to their cages where they were maintained on test diet until day 8. The receipt of tracer was designed as a crossover study. Piglets were randomly assigned to a tracer group, L-[1- ^{14}C]CYS or L-[1-^{14}C]MET. Piglet receiving L-[1- ^{14}C]CYS on day 6 received tracer L-[1-^{14}C]MET on day 8 and vice versa L-[1-^{14}C]MET not presented in this thesis, please see Shoveller, 2004).

6.1.5 Blood Sampling

During the oxidation period, blood (2 ml) was sampled every half hour via the femoral catheter. For amino acid analysis, 1.5 ml was placed in heparinized Vacutainers. For glutathione analysis 0.5 ml of blood was placed in a 1.5 ml Eppendorf tube containing 0.5 ml of stabilization solution 'A'(elucidated later in this chapter) (Jones et al., 1998). Both Vacutainers and Eppendorf tubes were then centrifuged to separate the red cells from the plasma. The plasma to be used for later amino acid analysis was then pipetted into 1.5 ml Eppendorf tubes and frozen in liquid nitrogen and stored at –80°C for later HPLC analysis. Of the treated glutathione plasma, 0.6 ml was transferred to a 1.5 ml Eppendorf tube containing 0.6 ml glutathione deproteinisation solution 'B' (10% w/v Perchloric Acid: Water) (Jones et al. 1998). These tubes were then frozen in liquid nitrogen and stored at –80°C for later HPLC glutathione analysis.

6.1.6 Necropsy

After the oxidation on day 8, piglets were anaesthetised with Isoflourane at 5 ml/min for sample collection. The liver and kidneys were removed, weighed and 2 g samples were collected, placed in 4 ml of Solution 'B' (10% w/v Perchloric Acid: Water) and frozen in liquid N_2 and stored at –80°C for later glutathione and protein analysis. A 40 cm length of medial jejunum was excised, rinsed and gently scraped. The removed mucosa was placed in 4 ml of Solution 'B', frozen in liquid N_2 and stored at –80°C for later glutathione and protein analysis. Piglets were killed by exsanguination.

6.1.7 Glutathione Analysis

6.1.7.1 Plasma

Glutathione analysis was accomplished via the procedure of Jones et al., (1998). Briefly, samples of blood were stabilized in solution 'A' a 100mM serine-borate (pH 8.5) containing (per ml) 0.5 mg sodium heparin, 1 mg bathophenanthroline disulfonate sodium salt (BPDS) and 2 mg iodoacetic acid. After treating with solution 'A', samples were centrifuged and the supernatant transferred to 1.5 ml eppendorf tubes containing solution 'B'. Solution 'B' was comprised of a 10% (w/v) perchloric acid solution with 0.2 M boric acid and 10 µM gamma-glutamylglutamate (internal standard). These tubes were centrifuged at 13000 rpms for 30 seconds and 300 µl of the supernatant was transferred into a 3 ml plastic test tube. Sixty microlitres of iodoacetic acid solution (7.4 mg/ml) was added and ~300 µl of saturated KOH/tetraborate solution added. Each sample was tested for pH 9 a

deviations from pH 9 interfere with HPLC analysis. Each sample was left for 20 minutes. Three hundred microlitres of dansyl chloride derivatizing agent (20mg/ml acteone) was then added to each sample. The samples were then covered in parafilm and left in a dark place at room temperature for 24 hours. After 24 hours 500µl of chloroform was added to each sample to extract any unreacted dansyl chloride. Samples were then covered in parafilm and stored in a dark cooler at 0-4°C until assay by HPLC.

Prior to HPLC analysis, derivatized samples were centrifuged at 3000 rpm for 2 min. An aliquot of the upper aqueous layer was transferred to the autosampler. One hundred microlitres of sample was injected and separation achieved utilizing a 3-aminopropyl column (5µm: 4.6mm x 25cm; supelcosil LC-NH_2, Bellefonte, PA. USA). HPLC solvent A was 80% methanol/water (v/v). Solvent B was an acetate-buffered methanol solution (pH 4.6) containing: 640 ml of methanol, 200 ml of stock solution (272 g sodium acetate trihydrate, 122 ml H_2O and 378 ml glacial acetic acid) 125 ml of glacial acetic acid and 50 ml of H_2O. The initial solvent conditions were 80% A, 20% B at 1 ml/min for 10 min. A linear gradient to 20% A: 80% B was achieved from time 10-30 min. These conditions were maintained until 46 min., after which the initial solvent conditions of 80% A, 20% B were restored.

Detection was achieved utilizing fluorometric detectors with monochromators set at 335 nm for excitation and 515 nm emission. Collection and liquid scintillation counting of radioactive fractions to determine specific radioactivity of plasma and

tissue glutathione and glutathione disulfide were carried out as described by House (1997a).

6.1.7.2 Tissue

Tissue samples collected at necropsy were homogenized for 2 minutes utilizing a polytron (Brinkman Instruments. Rexdale, ON, Can). The samples were then centrifuged at 3000 rpm for 15 minutes. The supernatant was then removed by pipette and used for glutathione analysis while the pellet was used for protein determination. Tissue supernatants were derivatized identically to that of plasma, described above, but utilized a 20 µl injection onto the column. The internal standard used in the plasma sample analysis, γ- glutamylglutamate, did not resolve from unknown peaks in the tissue samples. Therefore, to calculate the concentration of GSH present in each tissue sample serially diluted external standards of cysteine, GSH and GSSG were used.

6.1.8 Protein Determination

Protein was analysed utilising a Bicinchonic Acid assay kit (Sigma, BCA-1). Samples were analysed utilizing the 96 well-plate method and were read on a spectrometer (Molecular Devices Spectra Max 190, Sunnybrook, Ca. USA) at 560 nm.

6.1.9 Calculations

Tissue GSH concentrations were calculated as follows:

Concentrations were calculated utilizing an external curve generated separately for cysteine, glutathione and glutathione disulfide.

$[Tissue_{TGSH}$ (nmol/mg protein)] = ($[TGSH_{concentration}$ (nmol/l)] * 4 ml * g of ground sample) ÷ mg of protein

Incorporation of CYS into TGSH was calculated as follows:

$[Incorporation$ (dpm/umol/g)] = [($TGSH_{radioactivity}$ (dpm/20 µl)÷ [(TGSH $_{concentration}$ (nmol/l)/ g of ground sample)]]

6.1.10 Statistics

The data were analysed as a completely randomised cross-over design with CYS intake as the independent variable utilizing proc MIXED to identify differences among diets (SAS, V.8.0: SAS Institute, Cary, NC, USA). Weight was tested for covariance and was not significant for all datasets except for organ weight for which it was used as a covariate. Significant differences among treatments were tested utilizing Dunnetts t-test ($P < 0.05$). The dependent variables were: plasma and tissue total cysteine (cysteine + cystine, TCYS), tissue total glutathione (glutathione + glutathione disulfide, TGSH), tissue CYS incorporation into TGSH and glutathione (GSH), glutathione disulfide (GSSG) ratio (2GSH : GSSG). All variables were analysed using proc RSREG (SAS, V.8.0: SAS Institute, Cary, NC, USA) to define the response to dietary CYS. When the dependent variable was related to CYS

intake, the data was graphed (GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA).

6.2 Results

All animals were healthy over the course of the trial and none were treated with antibiotics beyond that outlined in the materials and methods section of this chapter.

6.2.1 Growth Data

Increasing dietary cysteine intake had no effect on the weight of the piglets for day 5-8 ($P > 0.05$, Table 5.1). In addition, average daily gain (ADG), calculated as: (weight at necropsy – weight start of experimental diet/ days on diet) was not affected by dietary CYS intake ($P > 0.05$, Table 5.1).

6.2.2 Organ Weights

Dietary CYS had no effect on the weight of the liver or kidney, either on an absolute basis or when expressed relative to body weight ($P > 0.05$, Table 5.2). As CYS inclusion in the diet increased to 0.1 g $kg^{-1}d^{-1}$ the weight of the mucosa increased ($P = 0.02$, Table 5.2). Once CYS inclusion increased beyond 0.1 g $kg^{-1}d^{-1}$ the weight of the mucosa decreased.

6.2.3 Tissue Protein Determination

There was no effect of increasing dietary cysteine intake on the protein content of the liver, kidney or jejunal mucosa (($P > 0.05$, Table 5.3).

6.2.4 Plasma Cysteine Concentrations

Increasing dietary cysteine intake was positively and linearly associated with the plasma concentration of cysteine ($P = 0.0021$, $R^2=0.31$, SEE =90.14, Figure 5.1). The quadratic relationship was not significant ($P = 0.07$, $R^2 = 0.09$, SEE = 86.04, Figure 5.1)

6.2.5 Tissue Cysteine Concentration

Dietary cysteine intake was positively and linearly associated to hepatic TCYS concentrations ($P < 0.0001$, $R^2 = 0.45$, SEE = 23.82, Figure 5.2). A quadratic relationship was tested and found not significant ($P = 0.50$, $R^2 = 0.01$, SEE = 26.40, Figure 5.4). However, there was no effect of increasing dietary cysteine intake on the concentration of renal TCYS ($P > 0.05$, Figure 5.3) or jejunal mucosa ($P > 0.05$, Figure 5.4).

6.2.6 Total Glutathione Concentration

6.2.6.1.1 Plasma

Plasma samples were derivatized and analysed by HPLC; however, plasma TGSH results are not reported because the detection limit (~1 nmol/l) was not sensitive for the low concentrations of plasma glutathione present.

6.2.6.1.2 Tissue

There was a linear and positive effect ($P = <0.0001$, $R^2 =0.45$, SEE =28.81, Figure 5.5) of increasing dietary cysteine intake on hepatic TGSH. A quadratic relationship

was tested and found non-significant (P = 0.90, R^2 = 0.0002, SEE = 36.62). However, there was no effect of dietary cysteine on renal or jejunal mucosa TGSH (P>0.05, Figure 5.6, 5.7). Hepatic TCYS was linearly and positively associated with hepatic TGSH (P = 0.02, R^2 = 0.18, SEE = 36.17, Figure 5.8). A quadratic relationship for increasing hepatic TCYS to affect hepatic TGSH was tested and found significant (P = 0.04, R^2 = 0.12, SEE = 34.03).

6.2.7 Incorporation of CYS into TGSH

Dietary cysteine intake did not affect the incorporation of CYS into hepatic or renal TGSH (P>0.05, Figure 5.9, 5.10). However, dietary cysteine tended to decrease to decrease the radioactivity of mucosal TGSH (P = 0.07, R^2 =0.21, SEE =2107, Figure 5.11). A quadratic relationship for increasing dietary cysteine affecting TCYS incorporation into mucosal TGSH was tested and found non-significant (P = 0.58, R^2 = 0.02, SEE = 397).

6.2.8 Glutathione to Glutathione Disulfide Ratio

As dietary cysteine intake increased so too did the concentration of hepatic GSH (P = 0.005, Table 5.3). There was no effect of increasing dietary cysteine intake on GSH or GSSG in either the kidney or jejunum (P> 0.05, Table 5.4, 5.5). There was a trend (P = 0.06, Table 5.5) of dietary cysteine intake to increase the concentration of GSSG in the liver. Dietary cysteine intake had no effect on the ratio of glutathione to glutathione disulfide in the liver or mucosa (P >0.05, Table 5.6). There was a trend

Table 5.1 Weights (g) on days 0, 5, 8 and Average Daily Gain (ADG, g) from day 5-8 of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1})

Diet[1]	0	0.05	0.10	0.15	0.20	0.25	0.40	0.50	Pooled SEM	P- value
n	4	4	4	4	4	4	4	4		
Wt 0[2]	1478	1547	1558	1580	1570	1540	1735	1414	147	0.19
Wt 5[2]	2232	2377	2350	2465	2322	2332	2450	2232	249	0.84
Wt 8[2]	2512	2685	2750	2881	2732	2674	3017	2552	331	0.46
ADG[2,3]	103.53	100.39	198.34	123.37	153.09	123.88	227.24	124.78	35.59	0.09

1. Dietary CYS intake (g kg^{-1} d^{-1})
2. All values reported as LSMEANS as calculated by proc MIXED
3. ADG calculated as: (wt day5 – wt day 8/3)

Table 5.2 Weights (g) of Tissues of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1}).

Diet[1]	0	0.05	0.1	0.15	0.2	0.25	0.4	0.5	Pooled SEM	P-value
n	4	4	4	4	4	4	4	4		
Liver Weight[2,5]	102.88	124.42	120.88	112.95	105.85	107.75	113.27	140.73	10.72	0.27
Liver Weight (g/kg BW)[5]	38	46	44	41	38	39	41	53	4	0.25
Kidney Weight[2,5]	25.30	21.97	20.71	20.22	18.49	20.64	20.75	22.81	1.63	0.23
Kidney Weight (g/kg BW)[5]	9.2	8.0	7.6	7.6	6.8	7.4	8.0	8.1	0.6	0.14
Mucosa Weight[3,4,5]	2.29^{b}	2.78^{ab}	3.35^{a}	3.07^{a}	2.40^{ab}	2.51^{ab}	3.01^{ab}	2.83^{abc}	0.29	0.02

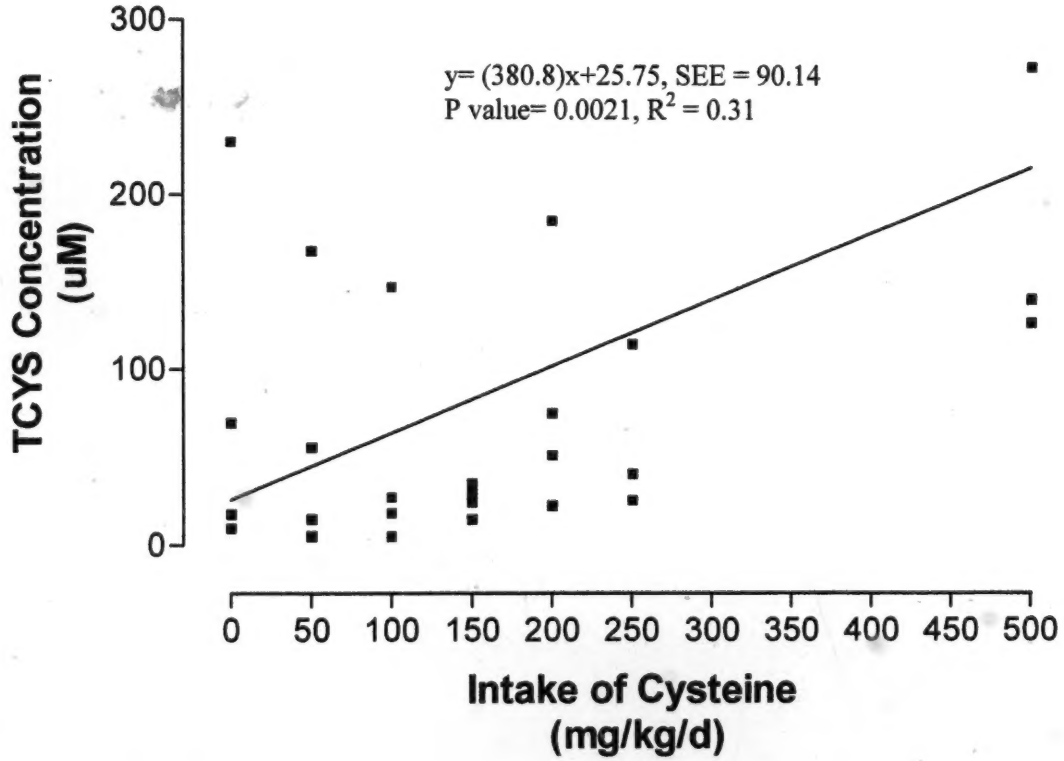
1. Intake of Cysteine (g kg^{-1} d^{-1})
2. Values used final body weight as a covariate
3. Letter differences within a row indicates significance ($P<0.05$, Dunnetts)
4. Weight of mucosa scraped from 40 cm length of jejunum
5. All values reported as LSMEANS as calculated by proc MIXED

Table 5.3 Protein Content (mg/g of tissue) of Organs and Tissues of Neonatal Piglets Receiving Enteral Nutrition with Increasi Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1}).

Diet[1]	0.0	0.05	0.10	0.15	0.20	0.25	0.40	0.50	Pooled SEM	P
n	4	4	4	4	4	4	4	4		
Liver (mg/g Tissue)[2]	32.35	30.91	27.77	30.71	31.32	32.94	33.43	37.57	3.22	
Kidney (mg/g Tissue)[2]	30.38	40.61	39.56	40.38	28.46	25.69	35.82	28.27	6.46	
Mucosa (mg /g Tissue)[2]	37.12	30.13	28.54	32.22	33.31	30.75	30.36	34.90	3.22	

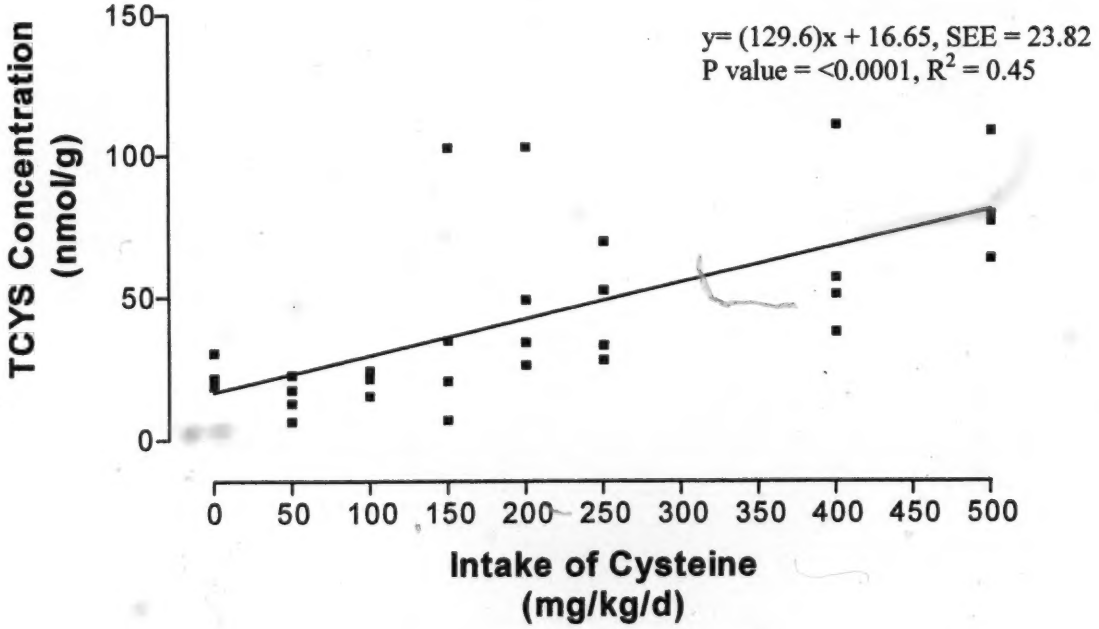
1. Intake of Cysteine (g kg^{-1} d^{-1})
2. All values reported as LSMEANS as calculated by Proc MIXED

Figure 5.1 Plasma Concentration of Total Cysteine (TCYS) (μM) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1}).



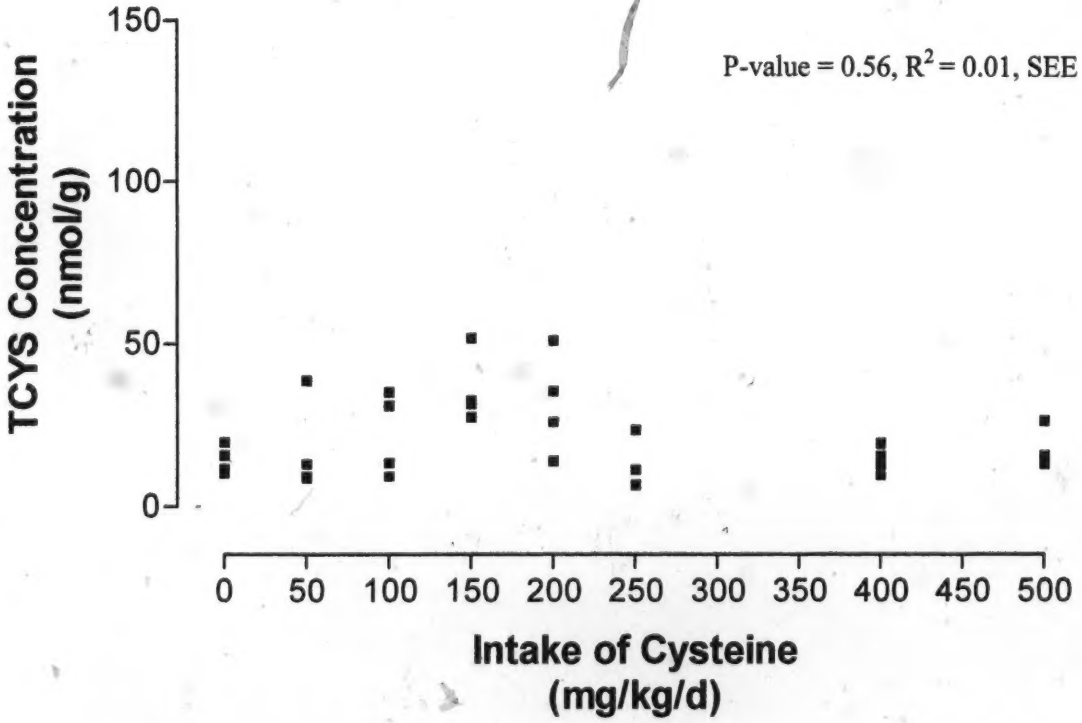
All data points individual graphed and analysed. One data point (diet 0.50 g/kg/d) is >300 uM.

Figure 5.2 Liver Concentration of Total Cysteine (TCYS) (nmol/g tissue) in Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1})



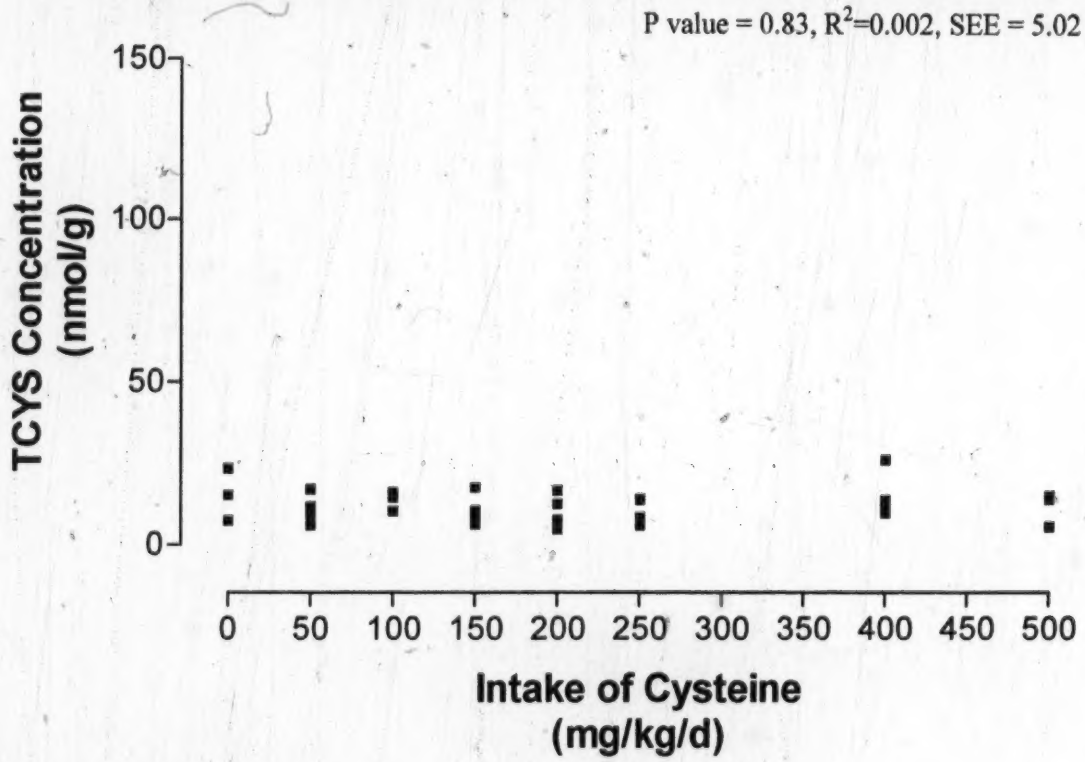
All data points individually graphed and analysed

Figure 5.3 Renal Concentration of Total Cysteine (TCYS) (nmol/g tissue) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1}).



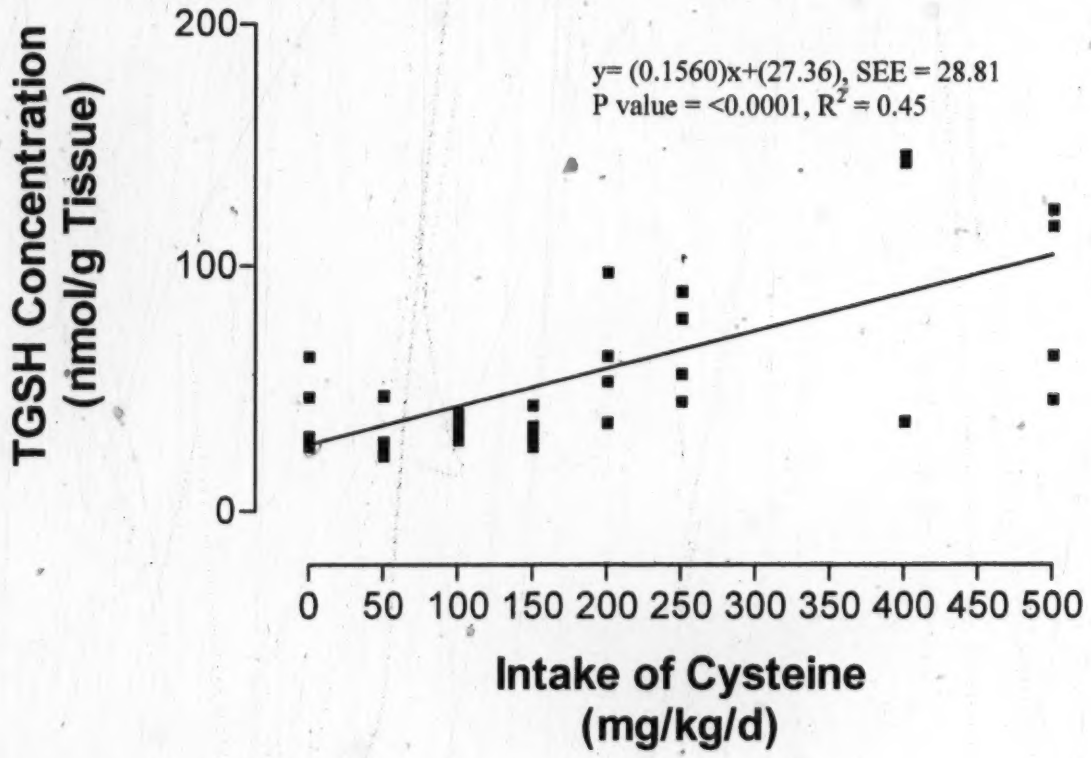
All data points individually plotted and analysed

Figure 5.4 Mucosal Concentration of Total Cysteine (TCYS) (nmol/g tissue) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)



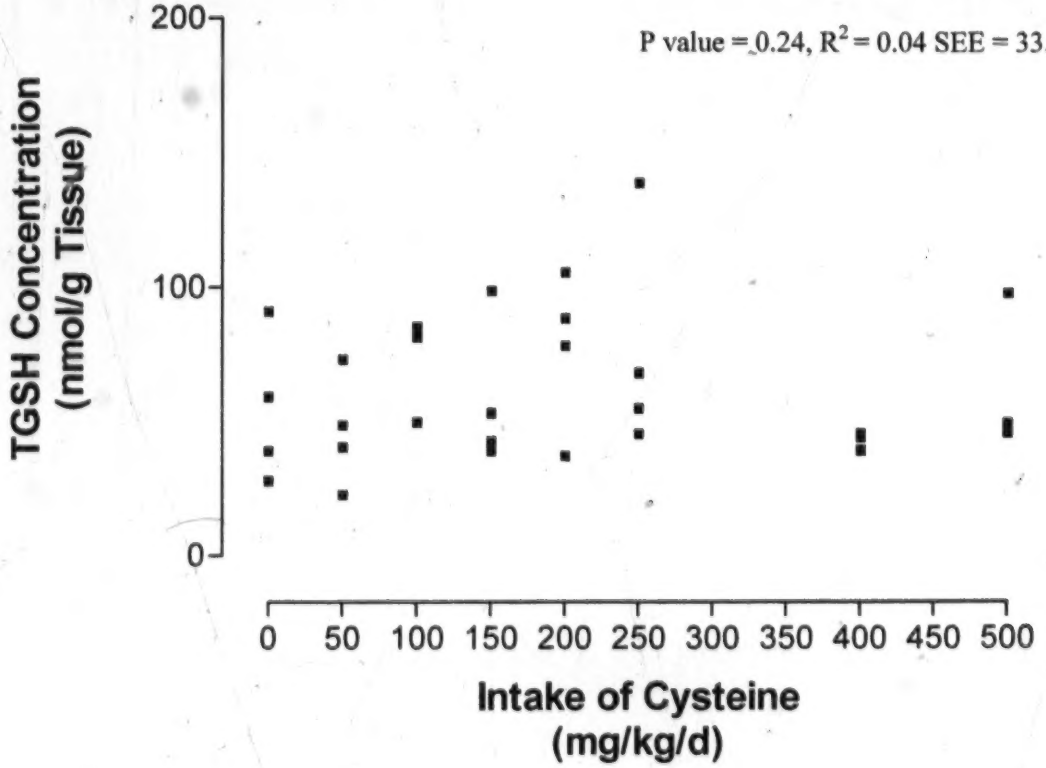
All data points individually graphed and analysed

Figure 5.5 Hepatic Total Glutathione (TGSH) Concentration (nmol/g tissue) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)


TGSH Concentration (nmol/g Tissue)
200
100
0
y= (0.1560)x+(27.36), SEE = 28.81
P value = <0.0001, R2 = 0.45
0 50 100 150 200 250 300 350 400 450 500
Intake of Cysteine (mg/kg/d)

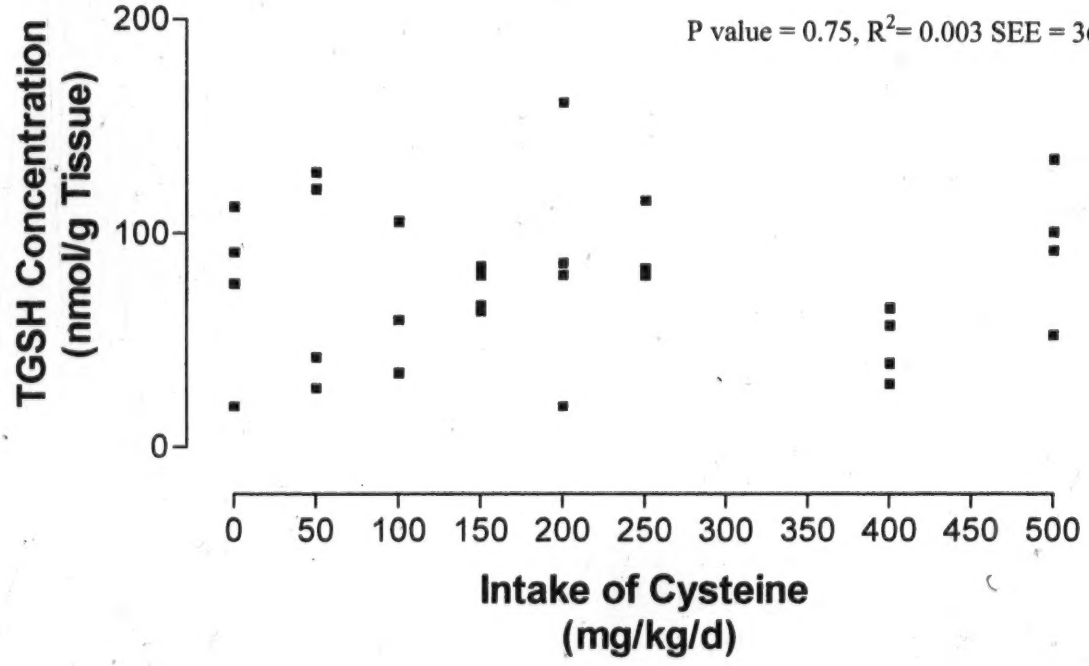
All data points individually plotted and analysed

Figure 5.6 Renal Total Glutathione (TGSH) Concentration (nmol/ g tissue) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g kg^{-1} d^{-1})


P value = 0.24, R^2 = 0.04 SEE = 33.85
TGSH Concentration (nmol/g Tissue)
200
100
0
0 50 100 150 200 250 300 350 400 450 500
Intake of Cysteine (mg/kg/d)

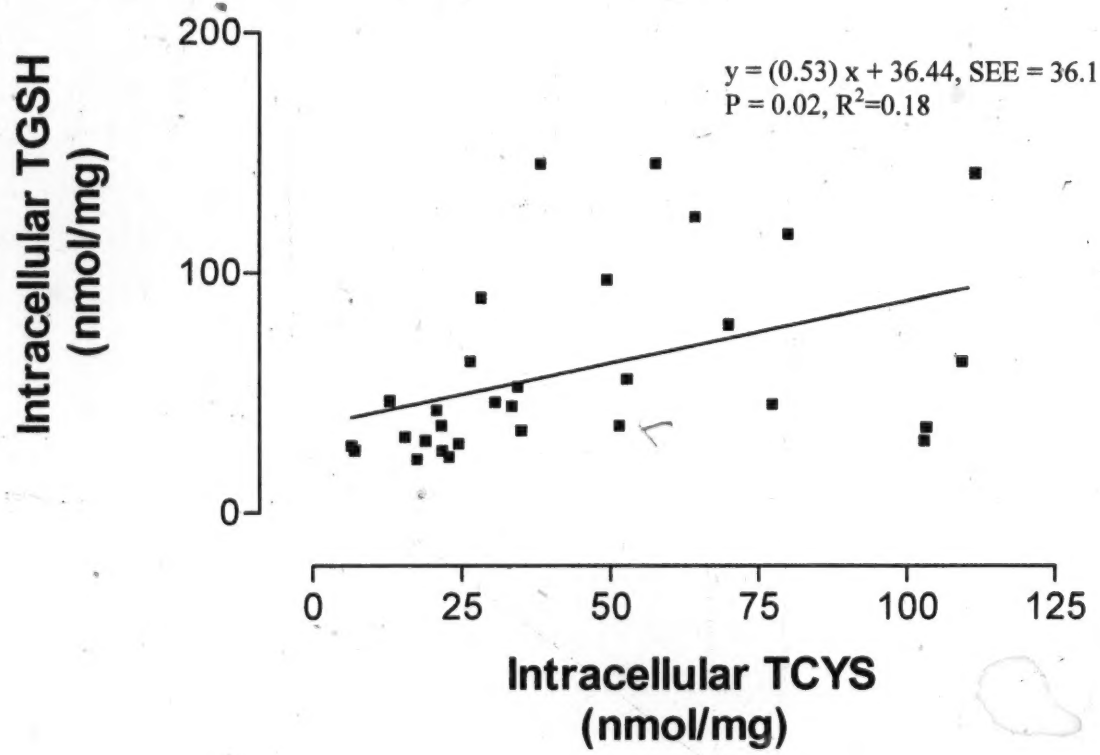
All data points individually plotted and analysed

Figure 5.7 Jejunal Mucosa Total Glutathione (TGSH) in the (nmol/g tissue) of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$).


P value = 0.75, R^2= 0.003 SEE = 36.03
TGSH Concentration (nmol/g Tissue)
200
100
0
0 50 100 150 200 250 300 350 400 450 500
Intake of Cysteine (mg/kg/d)

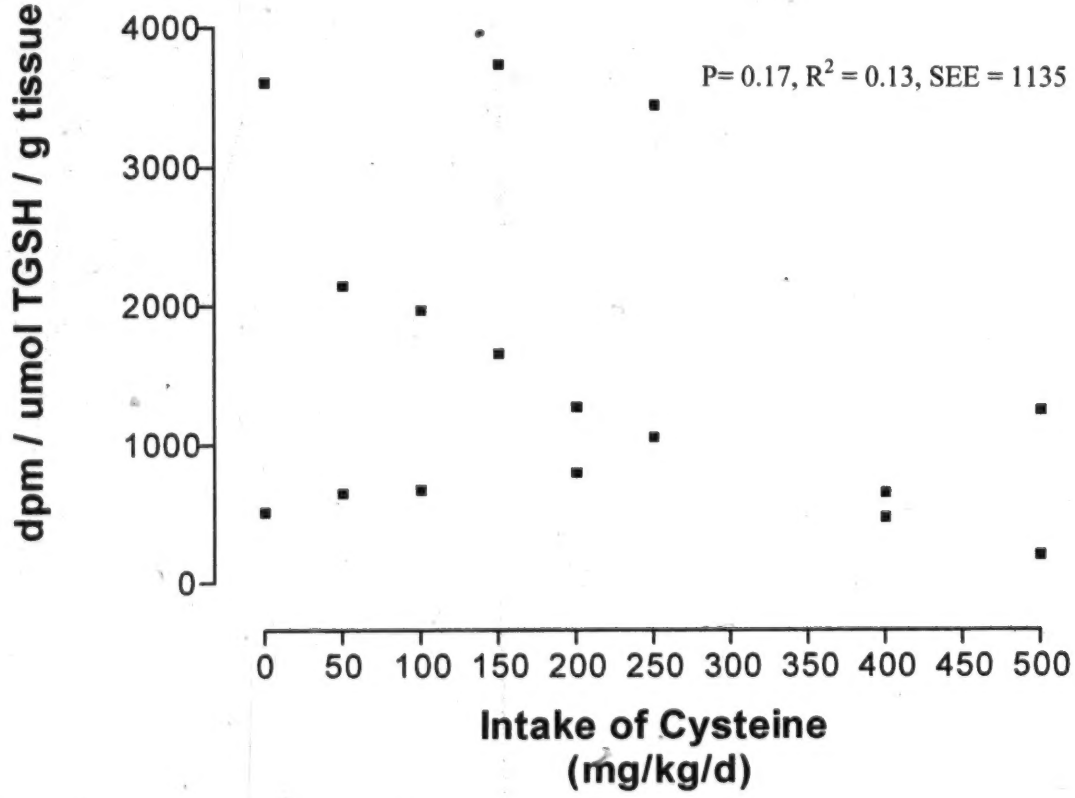
All data points individually plotted and analysed

Figure 5.8 Correlation of hepatic TCYS (nmol/g Tissue) Versus TGSH (nmol/g Tissue) of Neonatal Piglets Receiving Enteral Nutrition Containing Increasing Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)



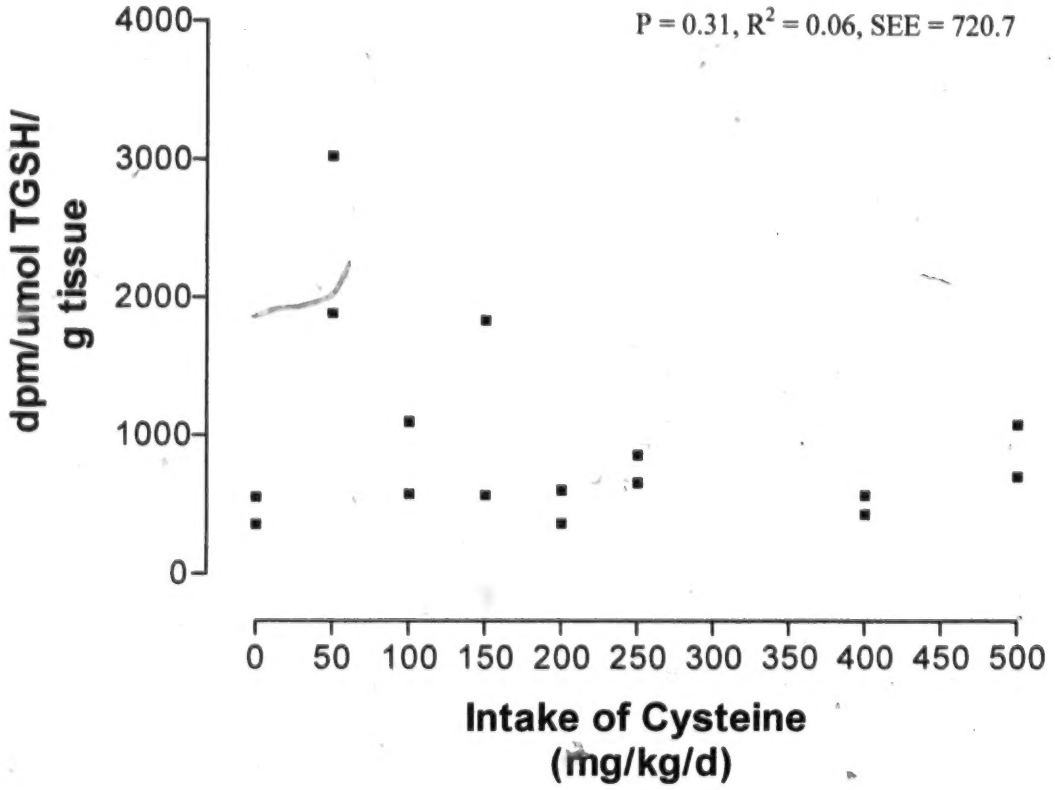
All data points individually plotted and analysed

Figure 5.9 Radioactivity (dpm/nmol) of Total Glutathione (TGSH, µmol) Per Gram Liver in of Neonatal Piglets Receiving Enteral Nutrition Containing Increasing Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$).



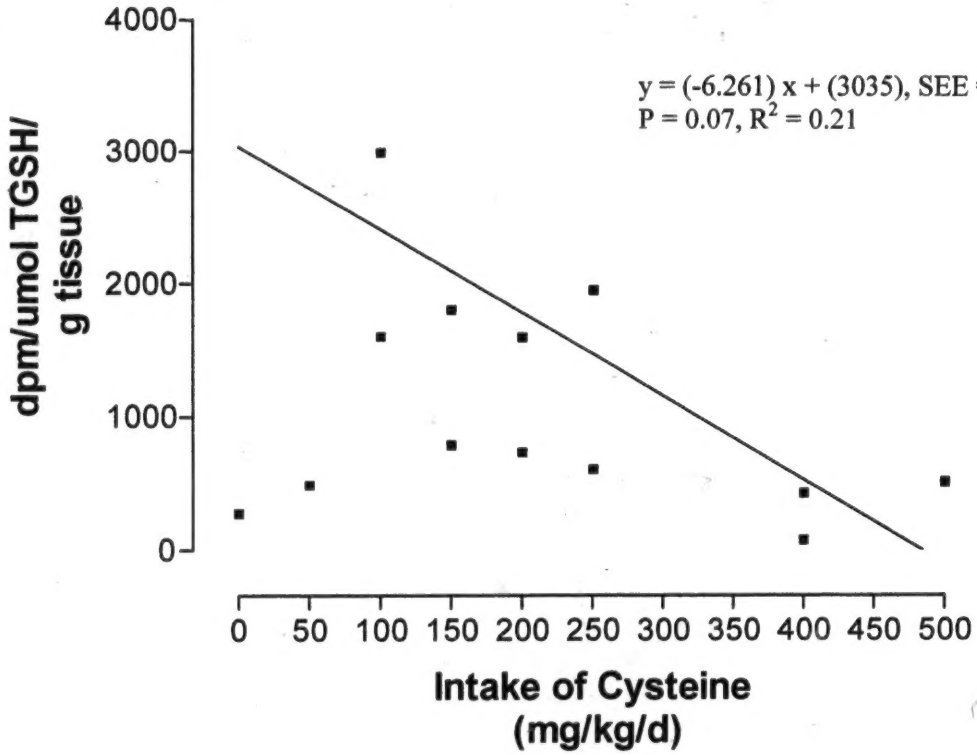
All data points individually plotted and analysed

Figure 5.10 Radioactivity (dpm/nmol) of Total Glutathione (TGSH, µmol) per Gram Kidney in Piglets After Receiving Enteral Nutrition Containing Graded Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)



All data points individually plotted and analysed

Figure 5.11 Radioactivity (dpm) of Total Glutathione (TGSH, µmol) Per Gram Jejunal Mucosa of Neonatal Piglets Receiving Enteral Nutrition Containing Increasing Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)



All data points individually plotted and analysed. Some data points lie outside 4000 dpm/umol

Table 5.4 Absolute Concentrations of Glutathione (GSH, µmol/g tissue) in the Liver, Kidney and Jejunum of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$).

Diet[1]	0	0.05	0.10	0.15	0.20	0.25	0.40	0.50	Pooled	P- value
n	4	4	4	4	4	4	4	4	SEM	
Liver[2,3,4]	25.37^{cd}	21.15^{d}	22.06^{d}	28.24^{bcd}	46.24^{bc}	33.99^{bcd}	95.10^{a}	60.76^{b}	9.47	0.0005
Kidney[2,5]	31.40	47.27	27.00	28.04	38.47	33.37	42.18	49.93	11.15	0.71
Mucosa[2]	37.52	54.56	31.74	33.89	35.93	60.30	25.99	35.61	13.63	0.64

1 Intake of Cysteine (g $kg^{-1}d^{-1}$)
2 All values reported as LSMEANS as calculated as proc MIXED
3 Letter differences within rows indicate significance ($P<0.05$) differences

Table 5.5 Absolute Concentration of Glutathione Disulfide (GSSG, µmol/g tissue) in the Liver, Kidney and Jejunum of Neonatal Piglets After Receiving Enteral Nutrition Containing Increasing Intakes of Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)

Diet[1]	0	0.05	0.10	0.15	0.20	0.25	0.40	0.50	Pooled	P-value
n	4	4	4	4	4	4	4	4	SEM	
Liver[2]	16.17	9.21	12.46	5.59	16.39	33.57	44.93	26.96	9.62	0.06
Kidney[2]	23.28	19.16	48.12	60.51	39.04	43.54	35.77	44.42	12.31	0.64
Mucosa[2]	37.39	35.46	27.30	40.26	51.13	30.63	22.38	60.19	11.99	0.33

1. Intake of Cysteine (g kg^{-1} d^{-1})
2. All values reported as LSMEANS as calculated by proc MIXED

Table 5.6 2GSH:GSSG Ratio in the Liver, Kidney and Jejunal Mucosa of Neonatal Piglets Receiving Enteral Nutrition with Increasing Intakes of Dietary Cysteine and Constant Methionine (0.25 g $kg^{-1}d^{-1}$)

Diet[1]	0	0.05	0.10	0.15	0.20	0.25	0.40	0.50	Pooled	P-
n	4	4	4	4	4	4	4	4	SEM	
Liver[2]	13.39	12.60	3.66	13.03	6.84	7.69	6.55	5.17	4.48	(
Kidney[2]	2.37	4.57	1.19	2.25	1.52	2.59	0.37	3.31	0.95	(
Mucosa[2]	2.45	1.59	2.57	3.01	3.14	2.03	2.32	1.28	0.89	(

1 Intake of diet (g $kg^{-1}d^{-1}$)
2 All values reported as LSMEANS as calculated by proc MIXED

(P = 0.06, Table 5.5) for increasing dietary cysteine intake to negatively affect the renal ratio of 2GSH: GSSG.

6.3 Discussion

In Experiment 1, increasing dietary CYS_2 beyond 50% of the MET intake and therefore decreasing the MET: CYS ratio of the early-weaned pig significantly decreased gastrointestinal mass and mucosal depth in the medial jejunum. We speculated that the decreased gastrointestinal mass and mucosal depth was due to increasing dietary intake of CYS_2 increasing the intracellular concentration of the anti-oxidant GSH, and that the increased GSH synthesis was exerting a mucolytic effect on the intestinal mucosa. The present experiment was designed to examine the effect of increasing dietary CYS intake and a decreasing MET: CYS ratio on the concentration of TGSH and the incorporation of CYS into hepatic, renal and jejunal mucosal TGSH. The experiment also permitted examination of the effects of increasing dietary CYS intake and decreasing MET: CYS ratio on intra-cellular redox status as determined by intra-cellular ratio of GSH to GSSG.

This study clearly demonstrates that increasing dietary CYS intake is positively and linearly associated with plasma TCYS concentrations (P=0.0021, Figure 5.1). These results are consistent with similar data in neonatal piglets. Shoveller et al. (2003b) found that dietary MET and TSAA intakes were positively and linearly associated with increasing plasma total cysteine in piglets receiving an oral elemental diet. Zlotkin et al. (1982) reported that infants fed CYS-supplemented parenteral diets had

significantly higher plasma cysteine concentrations compared to infants who received diets with no CYS but adequate TSAA. Thus, increasing dietary TSAA intakes results in increases in plasma CYS during both parenteral and enteral feedings.

Increasing dietary CYS was linearly and positively associated with hepatic TCYS concentrations ($P < 0.0001$, Figure 5.2) therefore as dietary CYS intake increased, the plasma TCYS increased and therefore increased whole body TCYS availability. We did not observe a change in the intracellular renal TCYS concentration with increasing dietary CYS intake ($P = 0.56$, Figure 5.3). Furthermore, there was no effect of dietary CYS intake on the concentration of TCYS in jejunal mucosal ($P = 0.83$, Figure 5.4).

Cystathionase (EC 4.4.1.1) and cystathionine synthase (EC 4.2.1.22), are the enzymes responsible for transsulphuration, and are present in the small intestine of the neonatal piglet (Finkelstein, 1998), but with specific activities of less than 10% of hepatic activity. Presently, it is unclear as to the maturation rate and the synthetic capacity of these enzymes to synthesize adequate amounts of CYS to meet TSAA requirements in the neonatal piglet. However, Zlotkin et al. (1981) reported that there was no increase of nitrogen retention in infants when CYS (0.77 g $kg^{-1}d^{-1}$) was supplemented to parenteral nutrition, and Shoveller et al., (2003b) found no differences in baseline oxidation of labelled phenylalanine used to measure MET requirement with or without CYS supplementation in gastrically fed piglets. These data indicate that there is no dietary requirement of CYS for protein synthesis in the neonatal piglet and that

CYS intake in the present experiment was sufficient to support protein synthesis and increasing plasma CYS concentrations in a linear fashion. While it is clear that increasing dietary MET, CYS or the TSAA concentration increases plasma TCYS concentrations in gastrically fed piglets, it is unclear as to the effects of these increased intakes on the products of CYS metabolism, namely GSH.

GSH is a tri-peptide that is synthesized through a variety of steps combining the amino acids glycine, glutamate and CYS (Meister and Anderson, 1983). Neither glutamate nor glycine are considered dietary indispensable amino acids because they are synthesized in vivo; however, CYS is conditionally essential because a lack of dietary MET will result in a lack of whole body CYS (Shoveller et al., 2003b). Therefore, CYS is the rate-limiting amino acid for the synthesis of GSH and as more CYS is made available via dietary sources, more hepatic GSH can be synthesized (Griffith, 1990). The liver is the primary site of GSH synthesis (~ 90% of whole body, Stipanuk et al., 1992) and the GSH synthesized in the liver is predominantly released into the plasma (~ 2/3) or into the intestine via the biliary duct (~1/3) (Dahm and Jones, 1994). Increasing dietary CYS intake was associated with a linear increase of hepatic TGSH concentration ($P = <0.0001$, Figure 5.5). Thus, it was the increased metabolic availability of CYS that promoted the observed increases in hepatic TGSH concentration ($P = 0.02$, Figure 5.8). A similar response was reported by Malloy and Rassin (1984) when supplemental CYS to the total parenteral diets of beagle pups, resulted in increased plasma and hepatic glutathione concentrations. Therefore, increased dietary CYS intake is related to increase hepatic TCYS that is

correlated, with an increase in hepatic TGSH concentrations. With the understanding that the liver is responsible for 90% of whole body GSH, increasing intakes of dietary CYS would likely increase whole body TGSH availability.

The kidney is not a major site of CYS synthesis or catabolism. GSH is however, broken-down to its constituent amino acids in the kidney via glutamyl transpeptidase (Meister and Anderson, 1983). As opposed to the liver, renal GSH depends primarily on extracellular GSH. When GSH is provided intravenously, renal GSH concentrations increase but, when equimolar concentrations of CYS are supplied, there is no change in renal GSH concentration. In fact, at high concentrations of intravenous CYS administered to rats there is a marked decrease in renal GSH concentrations (Aebi and Lauterberg, 1992). High concentrations and activities of γ-glutamyltransferases in the kidney are responsible for renal TGSH concentration. This would explain the lack of response of renal TGSH to increasing intakes of dietary CYS ($P = 0.24$, Figure 5.6), as compared to the liver. The activities of the γ-glutamyl cycle enzymes are much higher in the kidney than in the liver. However, the rate of utilization and synthesis is much lower in the kidney due to decreased amounts and activities of GSH synthetic enzymes (Sekura and Meister, 1974). In the present study the lack of response of renal TGSH concentration when dietary CYS intakes are increasing may be explained by increased γ-glutamylpeptidases. As dietary CYS intake increased no observed changes in TGSH concentrations were observed in the kidney (Figure 5.6). Stipanuk et al. (1992) reported that in the rat, the kidney takes up plasma GSH and releases a comparable amount of CYS into the

plasma. However, to the best of the authors' knowledge this is the first examination of a dose-response in the kidney to increasing dietary CYS intake. The present results indicate that regardless of the concentration of CYS or GSH supplied to the kidney by the renal artery, there was no increase in intracellular kidney TCYS or TGSH concentrations. It may be concluded that TGSH presented to the kidney via the renal artery was immediately converted to CYS that was then exported from the kidney into the plasma, tightly regulating intracellular renal CYS concentrations. The mechanism(s) for this regulation is currently unclear. Also, the release of CYS from renal GSH degradation may be responsible for the rise in plasma TCYS observed in the present study.

While there was an effect of increasing dietary cysteine intake on both liver TCYS and TGSH, there was no increase observed in the incorporation of CYS into TGSH ($P = 0.17$, Figure 5.9). Activity of two of the key enzymes involved in CYS metabolism, cysteine dioxgenase (CDO) (EC 1.13.11.20) and γ-glutamylcysteine synthetase (GCS) (EC 6.3.2.3), have been observed to change in response to diets containing different levels of protein or MET. As protein content or MET concentration of the diet increase, there is increased activity of CDO but a decrease in GCS activity (Bella et al., 1996a). Stipanuk et al. (2002b) reported a similar reciprocal response of CDO and GCS in the liver of rats when CYS was supplemented to a low protein diet. This may account for the lack of increase in hepatic TGSH synthesis observed in the present experiment (Figure 5.9). However,

because there was a linear increase in TGSH concentration in the liver there was likely an increase of TGSH exported from the liver into the bile.

There were no differences in the intracellular cysteine concentrations or total GSH concentrations of the jejunal mucosa. There was however a trend for increasing dietary CYS intake to decrease the rate of incorporation of CYS into mucosal TGSH ($P = 0.07$, Figure 5.11). Endogenous GSH arrives in the lumen of the gut via two methods. One is delivery via the biliary duct and the other is via synthesis in the enterocyte (Dahm and Jones, 1994). Approximately 1/3 of hepatic GSH is exported into the bile (Kaplowitz et al., 1986). Because the synthesis of TGSH in the liver increased in response to dietary CYS, the liver most likely exported more TGSH to the bile. However, GSH exhibits a negative feedback on the first step of the two-step enzymatic process of its own synthesis (Pastore et al., 2003). We speculate that as more GSH is released into the bile from the liver, less GSH may be synthesized in the enterocyte, due to negative biofeedback. We speculate that as dietary CYS became less limiting, TGSH degradation to CYS was down-regulated resulting in less TGSH being degraded and less demand for TGSH to be synthesized to replenish lost TGSH.

The ratio of 2GSH to GSSG was not affected by the increased intake of dietary cysteine for the liver, kidney or mucosa (Table 5.4). While there were linear responses of increasing dietary CYS on liver GSH and GSSG ($P = 0.001$, $P = 0.002$, Tables 5.4 and 5.5 respectively), the responses were similar. Thus both hepatic GSH and GSSG increased but in relation to each other throughout the experiment resulting

in no effect of dietary CYS intake on the ratio of 2GSH: GSSG. The lack of change in redox status with increasing dietary CYS intake indicates that excess dietary CYS does not affect the mechanism(s) for maintaining the redox status of the cell in the neonatal piglet. As GSH redox status, and therefore the redox status of the cell, is dependent upon maintaining the ratio of 2GSH: GSSG (Filomeni et al., 2002), perturbations in this ratio would indicate an inability of the cell to function normally. In the resting cell (multiple species and tissues), the average ratio is as much as 100:1 (2GSH:GSSG), however this ratio can drop to 10 or even 1:1 under various stress conditions such as surgical stress (Pastore et al., 2003), HIV (Gmunder and Droge, 1991) or cancer (Miller et al., 2002). Researchers speculate that in disease states low redox status decreases CD8+ proliferation and activity, ultimately decreasing immunity via decreased cytotoxic T lymphocyte activity. The low ratios observed in the present experiment (Table 5.5) suggest that the piglets were experiencing oxidative stress but it is impossible to isolate the exact cause of the very low values reported. However, it is important to note that reported ratio values are from human and rat studies. To the authors' knowledge, no data have been previously reported on the ratio of 2GSH:GSSG in the neonatal piglet.

5.7 Conclusion

Increasing dietary CYS intake resulted in increased plasma TCYS concentrations (Figure 5.1) and in hepatic intra-cellular TCYS and TGSH concentrations (Figure 5.2, and 5.5). However, there was no effect of increasing CYS intake on the incorporation of CYS into TGSH. Thus, the increased intake of dietary CYS may have led to

increased hepatic TGSH by sparing TGSH degradation and decreasing TGSH turnover.

There was no change in the incorporation rate of CYS into TGSH in the liver, kidney or mucosa (Figure 5.9, 5.10 and 5.11) when dietary CYS intake increased. However, when dietary CYS intake increases CDO increases and GCS decreases. As dietary CYS intake increases so too, does the activity of CDO but a concomitant decrease in the activity of GCS occurs (Stipanuk et al., 2002). As dietary CYS intake and the concentration of hepatic TGSH increased, mucosal TGSH synthesis was reduced and less GSH was degraded.

There was a trend ($P = 0.07$, Figure 5.10) for increasing dietary CYS intake to decrease the incorporation rate of CYS into mucosal TGSH. The incorporation rate of CYS into mucosal TGSH decreased and the mucosal TGSH concentrations were maintained as evidenced by Figure 5.11). The liver synthesizes 90% of whole body TGSH (Griffith, 1999), and approximately 30% of all hepatic TGSH is exported to the bile (Kaplowitz et al., 1986). Knowing that GSH exhibits negative inhibition on the first GSH synthetic enzyme GCS, it may then be speculated that as dietary CYS intake increased, more hepatic TGSH was exported into the bile and less mucosal TGSH was synthesized because of reduced degradation.

There was no significant effect of increasing dietary CYS intake on the ratio of 2GSH:GSSG in the liver, kidney or jejunal mucosa. In the liver, it is unclear as to

why increasing CYS intake resulted in increased TGSH synthesis but did not translate into an improvement in redox status. In addition, the reported ratios were very low as compared to published values in the rat and in humans. These low values have been observed in the past during oxidative stress as seen in disease states (Pastore, et al., 2003). However, as these values are novel, making comparison with existing data regarding humans and rats may be inappropriate. To the authors' knowledge, this is the first estimate of tissue redox status in the neonatal piglet and neonatal piglets may inherently have lower oxidative status. Further examination of the effects of dietary CYS on the oxidative status of piglets is necessary in order to infer the significance of these reported values.

7.0 General Summary and Future Directions

7.1 General Summary

The main objective of the present research was to examine the effects of the total sulphur amino acids (TSAA), methionine (MET) and cysteine (CYS), on the growth and gastrointestinal development of the early-weaned piglet. In modern weaned piglet diets, the feedstuffs used are generally low in the TSAA and often result in TSAA being the 2nd or 3rd limiting amino acid in the diet. In an effort to formulate diets that meet the NRC (1998) requirement for TSAA in 3-5 kg piglets, nutritionists supplement crystalline free MET or try to include feedstuffs that will raise the concentration of MET or CYS in the diet. MET can meet 100% of the TSAA requirement (Chung and Baker, 1992a) but many of the highly digestible feedstuffs utilized in diet formulation contain lower ratios of MET to CYS_2, which may result in CYS_2 being present at concentrations greater than 50% of the TSAA content. Due to this amino acid profile, the concentration in a weaning diet can be greater than the recommended ratio of 1:1 (NRC, 1998). Also, the MET requirement for 3-5 kg piglets receiving an elemental diet was recently determined to be 0.42 g/kg/d (Shoveller et al., 2003a) not 0.50 g/kg/d as recommended by NRC (1998). With the understanding that the requirement for the TSAA may be lower than currently used in formulation and that common feedstuffs used in weaning diets often result in high intakes of CYS relative to MET an experiment was designed to examine the effect of various TSAA concentrations and ratios on growth and gastrointestinal development of early-weaned piglets.

In Experiment 1, piglets that received 50% of the TSAA requirement (Diet 1, 0.25 g/kg/d)) or 75% of the TSAA requirement (Diet 2, 0.38 g/kg/d) with a ratio of 1:2 MET: CYS_2 had decreased growth, nitrogen balance and jejunal mucosal weights and total mucosal depths than their more adequately fed counterparts. MET is considered an essential amino acid because mammals cannot synthesize it endogenously (Lehninger et al. 1993). When MET was limiting, as in the cases of diets 1 and 2, the potential for growth was not achieved. The poor performance overall of piglets receiving these diets (1 and 2) was expected. What was not expected was the equal performance of the piglets receiving diet 3 (75% of the TSAA requirement containing a ratio of 2:1 MET: CYS_2) compared to all diets we deemed adequate. Overall, the animals that received diet 3 (0.38 g/kg/d TSAA) performed as well or better than animals receiving diets that contained the recommended (NRC, 1998) concentration and ratio of TSAA (diet 4: 0.50 g/kg/d). These data support our previous work (Shoveller et al., 2003) that concluded that the requirement for TSAA in 3 kg piglets is lower than recommended by NRC (1998). Similar growth rates, nitrogen retention and gastrointestinal development across the adequate diets was expected for piglets receiving diets 4, 5 (150% TSAA 1:2 MET: CYS_2, 0.75 g/kg/d), 6 (150% TSAA 2:1 MET: CYS_2, 0.75 g/kg/d), and diet 7 (200% TSAA 1:1 MET: CYS_2, 1.0 g/kg/d) but not for those piglets that received diet 3. Due to the low TSAA content these animals were expected to grow more slowly, have lower nitrogen retention and have histological intestinal values similar to those on the MET-deficient diets.

A pattern emerged from the gastrointestinal data regarding the ratio of MET: CYS_2. As the concentration of CYS_2 in the diet increased beyond 50% of MET inclusion in the diet a decrease in mucosa weight and total depth occurred. With each additional increase of dietary CYS_2 within a MET group (25%, 50% and 100% methionine), a concomitant decrease in the jejunal mass and total depth was observed. The repetitive nature of this pattern throughout a large proportion of the datasets indicated that there was a negative effect of excess dietary CYS_2 on growth and the gastrointestinal tract. This is of concern as early-weaned piglets are removed from their dam at <21 days, immediately mixed with non-littermates and exposed to a new environment and dietary source. These animals often experience a "growth lag" due to these stressors, lasting from 3 days to 1 week (Williams, 2003). During this period, feed intake is reduced forcing piglets to rely on limited body stores. This period of adjustment requires piglets to adapt to consuming a solid diet. Consumption of solid feed is often accompanied by intestinal inflammation (Hampson, 1986). Decreased mucosal depth coupled with dietary induced inflammation may act to lengthen the growth depression observed at weaning. Curiously sows' milk, the diet that early-weaned piglets are adapted to consume, contains a ratio of 2:1 MET: CYS_2. Providing rations that more accurately reflect diets that the piglet is adapted to consume that is, to reflect sows milk, may help to reduce the length or severity of the weaning growth depression. Further, providing such diets may lead to increased gastrointestinal health. A healthy, adapted gastrointestinal tract may allow piglets to grow more quickly, efficiently and reduce the number of days to market.

The question of why increased CYS_2 decreased the gastrointestinal mass and depth in the early-weaned piglet required further investigation. We speculated that the increased dietary CYS_2 resulted in subsequent increases in mucosal glutathione (GSH) synthesis and concentration. In cystic fibrosis, N-acetylcysteine, a precursor of GSH via CYS is administered as a mucolytic agent (Rubin, 2002). The –SH group of GSH, binds with CYS moieties in the mucus lining the bronchial tubes and lungs. This binding alters the structure of the mucus thereby increasing the fluidity and increasing the ease with which patients can expel the mucus (Rubin, 2002).

Increased dietary CYS intake increases plasma GSH concentration (Jahoor et al., 1995) but it is unclear if a similar effect occurs in the gut when increasing dietary CYS is provided. Experiment 2 investigated diets, containing a constant concentration of MET but graded increases of CYS were fed to gastrically catheterised piglets for 3 days. Piglets then underwent a 6 h oxidation utilizing L- [1-^{14}C] CYS. After oxidation on day 8, piglets were anaesthetised, tissue samples collected and the piglets were euthanised. The tissues sampled allowed investigation of the effect of increasing dietary CYS intake on the TGSH concentration and incorporation rate of CYS into tissue TGSH.

There was a positive linear association between dietary CYS and liver intracellular concentrations of free TCYS. The liver is the primary site of sulphur amino acid metabolism and increased intracellular free TCYS would result as the metabolic

availability of CYS increased. A similar relationship was observed for dietary CYS and intracellular hepatic TGSH concentrations. Hepatic TCYS concentration and TGSH concentration were positive and linearly associated (P = 0.0021, Figure 5.8) indicating that increased dietary cysteine intake increased hepatic TGSH synthesis. Surprisingly, there was no change in the incorporation of CYS into hepatic TGSH. Regardless of the dietary concentration of CYS, the rate of CYS incorporated into hepatic TGSH remained relatively constant (Figure 5.9). Bella et al., (1999) reported that increased MET or protein intake positively increased CDO activity and that a reciprocal decrease in GCS activity occurred. Stipanuk et al., (2002) reported a similar response for increasing dietary CYS intake. As dietary CYS intake increased there was a positive increase in CDO activity but a concomitant decrease in GCS activity. This decreased activity was most likely responsible for the lack of increase in the rate of incorporation of CYS into hepatic TGSH.

The kidney showed neither an increase in intracellular TCYS or TGSH concentration due to increased dietary CYS intake. TGSH is exported from the liver via the plasma to the kidney. The kidney contains high concentrations of γ-glutamyltransferase, an enzyme responsible for breaking GSH into its constituent amino acids for transport into the cell (Meister, 1983). Stipanuk et al., (1992) reported that equivalent amounts of CYS were transported away from the kidney, for equal amounts of GSH delivered. While CYS balance to and from the kidney was not measured, it may be assumed that for each absolute increment of GSH exported to the kidney from the liver, a similar increment of CYS was transported away from the kidney.

In the gastrointestinal tract no significant increase in either intracellular TCYS or TGSH concentration occurred. There was also no affect of increasing dietary CYS intake on the incorporation of CYS into TGSH in the jejunal mucosa. Endogenous lumenal GSH is derived from two sources, hepatic export via the biliary duct and mucosal synthesis (Dahm and Jones, 1998). Because the liver increased the absolute amount of TGSH synthesised, in response to increased dietary CYS intake, more GSH would be exported into the lumen. GSH exhibits negative feedback on the first enzyme, glutathione synthetase, of the glutathione synthetic pathway (Anderson, 1998). The negative feedback mechanism may have inhibited mucosal TGSH synthesis, and thus the concentration of mucosal TGSH was maintained.

The results of Experiment 1 and 2, demonstrated an effect of CYS on the gastrointestinal tract of the early-weaned piglet. Also, there may be a link between the trend for decreased incorporation of dietary CYS into mucosal TGSH observed in Experiment 2 and the decreased mucosal weight and depth observed in Experiment 1. We speculate, that as dietary CYS intake increased, the gastrointestinal mucosa was exposed to a greater oxidative load via CYS oxidation. This may explain the both the low redox values reported in Experiment 2 and the decreased total depth in the jejunal mucosa reported in Experiment 1. Because the oxidative load was increased, the redox status of the cell was decreased and thus would have resulted in cellular oxidative injury. Without GSH synthesis in the mucosal cells (trend for decreasing incorporation of CYS into mucosal TGSH (P=0.07)) products of oxidation such as

free radicals and superoxides would damage the cell leading to increased cellular death. Increased cellular apoptosis would in turn stimulate cytokine production and a subsequent inflammatory response leading to a 'leaky gut' and thus decreasing the effectiveness of the small intestine as a barrier and allowing bacterial translocation to occur (Miller et al., 2002). However, due to the lack of literature data regarding the redox status of neonatal piglets, or measurements of the permeability of the small intestine due to increasing intakes of dietary CYS, inferring significance from the current experiments is difficult.

7.2 Future Directions

From both Experiments 1 and 2, many possible future research options exist. In order to fully examine the effects of dietary CYS on gastrointestinal mucosa, the effect of a wider range in dietary CYS at fixed MET intakes must be investigated. Diets with higher CYS concentrations and higher ratios of CYS to MET would allow researchers to determine if CYS should be supplemented or not to diets of early-weaned piglets to meet the TSAA requirement to ensure gastrointestinal health. Further, diets utilizing supplemented MET and CYS would allow investigation into GSH synthesis and incorporation of endogenous CYS. If confirmed, the mechanism of CYS toxicity should be investigated, and it is important to determine whether increasing dietary CYS increased gut permeability. Studies investigating the response of mucosal weight, total depth and permeability to TSAA supplementation would indicate whether there is a detrimental effect to feeding CYS above 50% of MET inclusion into diets. Increased permeability would decrease the effectiveness of the intestine as

a barrier. In order to determine what exactly is occurring in the small intestine when increasing dietary CYS is available, CYS incorporation into mucin and the quantity of CYS and GSH entering the portal vein should also be examined. Increasing CYS incorporation into mucin may explain the decrease in mucosal mass and depth observed in Experiment 1 by affecting the viscosity of the protective lining of the gut. Catabolism and utilization of CYS and transport of CYS and metabolites into the portal vein should also be determined.

Understanding the role of dietary CYS in the small intestine will help nutritionists formulate diets that meet the specific requirements of the early-weaned piglets. These diets will encourage gastrointestinal adaptation and health in the piglet thereby increasing the profitability of segregated early-weaning in modern agriculture.

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9.0 Appendix: Data tables from Experiment 1. Supplementation of Sulphur Amino Acids

Appendix 1. Mean Daily Weights of Piglets Consuming Experimental Diets Containing Various Concentrations of the Sulphur Amino Acid for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
4[2]	3.79	3.93	4.00	3.91	3.92	0.05	0.11
5[3]	3.97^{c}	4.07^{bc}	4.22^{a}	4.11^{b}	4.05^{bc}	0.05	0.01
6	4.11^{c}	4.24^{b}	4.37^{a}	4.27^{ab}	4.23^{b}	0.05	0.03
7	4.19^{c}	4.34^{b}	4.43^{a}	4.41^{ab}	4.35^{ab}	0.06	0.01
8	4.28^{c}	4.46^{b}	4.72^{a}	4.51^{b}	4.48^{b}	0.07	0.002
9	4.35^{c}	4.61^{b}	4.80^{a}	4.72^{ab}	4.66^{ab}	0.07	0.0004
10	4.44^{c}	4.72^{b}	5.03^{a}	4.79^{b}	4.83^{b}	0.10	0.004

1. Diets are represented as a percentage of NRC (1998) requirement; TSAA = total sulphur amino acid intake
2. All values represented as LSMEANS as calculated by proc MIXED
3. Letter differences within a row indicate ($P > 0.05$, Dunnett) significance

Appendix 2. Mean Daily Weights of Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of the Sulphur Amino Acid for 7 days

Diet[1] TSAA	25-25 50	25-50 75	50-25 75	50-50 100	50-100 150	100-50 150	100-100 200	Pooled SEM	P-value		
									MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
4[2]	3.79	3.91	3.94	4.00	3.93	3.92	3.89	0.05	0.12	0.24	0.71
5[3]	3.97^{c}	4.03^{bc}	4.11^{b}	4.23^{a}	4.11^{b}	4.11^{b}	4.06^{b}	0.05	0.006	0.12	0.73
6	4.09^{c}	4.21^{b}	4.26^{b}	4.37^{a}	4.26^{b}	4.27^{ab}	4.24^{b}	0.05	0.03	0.10	0.74
7	4.19^{b}	4.32^{ab}	4.35^{a}	4.44^{a}	4.23^{b}	4.41^{a}	4.34^{ab}	0.06	0.03	0.60	0.82
8	4.28^{c}	4.41^{b}	4.51^{b}	4.72^{a}	4.49^{b}	4.54^{b}	4.49^{b}	0.07	0.003	0.02	0.34
9	4.35^{c}	4.56^{b}	4.66^{b}	4.81^{a}	4.74^{a}	4.70^{ab}	4.68^{ab}	0.07	0.004	0.04	0.79
10	4.42^{b}	4.56^{b}	4.87^{ab}	5.03^{a}	4.74^{b}	4.88^{ab}	4.83^{ab}	0.10	0.0005	0.11	0.44

1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3. Letter differences within rows indicates significance ($p<0.05$, Dunnets) for MET, CYS and CYS(MET). Body weight on day 3 and previous day feed intake used as a covariate

Appendix 3. Daily Nitrogen Balance for Piglets Consuming Experimental Diets Containing Various Concentrations of Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	P-value TSAA
n	6	12	6	12	6		
Day 4[2]	-12	84	86	83	78	35	0.17
5[3]	69[b]	72[ab]	81[ab]	78[ab]	83[a]	3	0.02
6	69	75	82	81	75	3	0.08
7	71	71	79	76	78	4	0.41
8	70	76	70	81	80	4	0.13
9	68	74	86	79	67	5	0.10
10	73[b]	75[b]	88[a]	84[ab]	81[ab]	3	0.002

1. Diets are represented as a percentage of NRC (1998) requirement; TSAA = total sulphur amino acid intake
2. All values are reported as LSMEANS as calculated by proc MIXED
3. Letter differences within a row indicate (P > 0.05, Dunnetts) significance

Appendix 4. Daily Nitrogen Balance for Piglets Consuming Experimental Diets Containing Various Concentrations and Ratios of Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100				
TSAA	50	75	75	100	150	150	200	Pooled SEM	MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Day 4[2]	-12	84	84	86	81	86	78	35	0.40	0.40	0.39
5[3]	69^{ab}	66^{b}	79^{ab}	81^{ab}	82^{ab}	73^{ab}	83^{a}	3	0.01	0.09	0.21
6	69^{ab}	67^{b}	82^{a}	82^{a}	82^{a}	80^{ab}	75^{ab}	3	0.001	0.86	0.67
7	71^{ab}	64^{b}	79^{a}	79^{a}	80^{a}	71^{ab}	78^{ab}	4	0.01	0.21	0.52
8	70	73	80	70	86	78	79	5	0.44	0.22	0.18
9	68^{b}	67^{b}	82^{a}	87^{a}	79^{a}	79^{a}	67^{b}	5	0.005	0.32	0.78
10	73^{b}	68^{c}	81^{a}	87^{a}	83^{a}	85^{a}	82^{a}	2	<0.0001	0.58	0.06

1 Diet: methionine: cystine ratio relative to requirement (100); MET = methionine intake, CYS = cystine intake,CYS(MET)=cystine intake within a methionine intake

2 Values are LSMEANS as calculated by proc MIXED

3 Letter differences within a row indicates significance (P<0.05, Dunnetts) for MET, CYS and CYS(MET)

Appendix 5. Average Villus Height (mm) for Intestinal Segments from Piglets Consuming Experimental Diets Containing Various Concentrations of Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	TSAA
n	6	12	6	12	6		
Duodenum[2]	0.34	0.31	0.40	0.36	0.40	0.03	0.12
Medial Jejunum	0.28	0.33	0.31	0.31	0.29	0.03	0.88
Medial Ileum	0.29	0.33	0.36	0.37	0.37	0.03	0.20

1. Diets are represented as a percentage of NRC (1998) requirement; TSAA = total sulphur amino acid intake
2. All values reported as LSMEANS as calculated by proc MIXED

Appendix 6. Average Villus Height (mm) for Intestinal Segments from Piglets Consuming Experimental Diets Containing Various Concentrations of Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100				
TSAA	50	75	75	100	150	150	200	Pooled SEM	MET	CYS	CYS(MET)
n	6	6	6	6	6	6	6				
Duodenum[2,3]	0.34^{ab}	0.33^{b}	0.28^{b}	0.40^{a}	0.43^{a}	0.31^{b}	0.40^{a}	0.03	0.05	0.13	0.12
Medial Jejunum	0.28	0.27	0.38	0.31	0.31	0.29	0.27	0.03	0.06	0.40	0.61
Medial Ileum	0.29^{b}	0.31^{b}	0.36^{a}	0.36^{a}	0.34^{ab}	0.39^{a}	0.37^{a}	0.03	0.04	0.73	0.92

1. Diet: methionine: cystine ratio relative to requirement (100)
2. All values expressed as LSMEANS as calculated by proc MIXED; MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake
3. Letter differences within a row indicate significance ($P<0.05$, Dunnetts) for MET, CYS and CYS(MET)

Appendix 7. Muscle Amino Acids (μmol/g wet weight) of Piglets Consuming Various Concentrations of the Sulphur Amino Acids for 7 days

Diet[1]	50	75	100	150	200	Pooled SEM	TSAA
n	6	12	6	12	6		
Indispensable							
Histidine[2]	0.22	0.20	0.19	0.25	0.33	0.04	0.12
Isoleucine	1.85	0.63	0.46	0.59	0.48	0.44	0.11
Leucine	3.72	1.40	1.36	1.45	1.43	0.86	0.18
Lysine	0.72	0.59	0.24	0.18	0.31	0.22	0.25
Methionine	0.58	0.64	0.79	1.04	0.89	0.22	0.23
Phenylalanine	0.23	0.39	0.65	0.57	0.64	0.29	0.15
Threonine	2.17	1.95	2.90	2.75	2.63	0.36	0.11
Tryptophan	1.27	1.07	1.55	0.97	0.23	0.59	0.54
Valine	0.81	0.71	0.80	0.81	0.66	0.09	0.56
Dispensable							
Alanine	1.33	1.43	1.88	2.32	1.79	0.52	0.42
Arginine	3.19	2.29	2.94	2.33	2.07	0.50	0.35
Aspartate	2.68	2.67	1.81	3.88	2.29	0.89	0.25
Cystine	0.82	0.30	0.38	0.42	1.45	0.43	0.22
Glutamate	0.12	0.12	0.27	0.38	0.15	0.18	0.59
Glutamine	4.28	3.44	4.59	4.14	3.39	0.81	0.65
Glycine	1.29	1.58	1.13	1.93	1.00	0.75	0.83
Hydroxyproline	0.32	0.31	0.47	0.44	0.25	0.16	0.64
Proline	0.72	0.65	0.80	0.99	1.09	0.26	0.50
Serine	0.14	0.16	0.09	0.18	0.07	0.06	0.62
Taurine	8.18	8.40	11.61	13.14	11.99	2.03	0.06
Tyrosine	1.02	0.93	1.21	1.24	1.04	0.29	0.81

1. Diets are represented as a percentage of NRC (1998) requirement; TSAA = total sulphur amino acid
2. All values reported as LSMEANS as calculated by proc MIXED

Appendix 8. Muscle Amino Acids (μmol/g wet weight) of Piglets Consuming Various Concentrations and Ratios of the Sulphur Amino Acids for 7 days

Diet[1]	25-25	25-50	50-25	50-50	50-100	100-50	100-100	Pooled	MET	CYS	CYS(MET)
TSAA	50	75	75	100	150	150	200	SEM			
n	6	6	6	6	6	6	6				
Indispensable											
Histidine[2]	0.22	0.19	0.21	0.19	0.24	0.27	0.33	0.04	0.13	0.41	0.98
Isoleucine	1.85	0.82	0.43	0.46	0.62	0.55	0.48	0.44	0.15	0.57	0.40
Leucine	3.72	1.41	1.40	1.36	1.41	1.49	1.43	0.86	0.42	0.43	0.39
Lysine	0.72	0.93	0.25	0.24	0.12	0.24	0.31	0.22	0.06	0.93	0.76
Methionine	0.58	0.51	0.77	0.79	1.15	0.92	0.89	0.22	0.11	0.98	0.53
Phenylalanine	0.23	0.39	0.39	0.65	0.65	0.48	0.64	0.29	0.57	0.48	0.13
Threonine	2.17	1.89	2.01	2.90	2.70	2.80	2.63	0.36	0.28	0.51	0.19
Tryptophan	1.27	0.43	1.71	1.55	0.73	1.21	0.23	0.59	0.31	0.14	0.79
Valine	0.81	0.70	0.73	0.80	0.71	0.91	0.66	0.09	0.60	0.22	0.29
Dispensable											
Alanine	1.33	1.25	1.60	1.88	1.73	2.90	1.76	0.52	0.13	0.52	0.53
Arginine	3.19	2.44	2.14	2.94	2.08	2.58	2.07	0.50	0.83	0.55	0.28
Aspartate	2.68	2.51	2.82	1.81	4.11	3.64	2.29	0.89	0.97	0.83	0.10
Cystine	0.82	0.34	0.26	0.38	0.58	0.26	1.46	0.43	0.20	0.54	0.48
Glutamate	0.12	0.10	0.14	0.27	0.14	0.62	0.15	0.18	0.22	0.29	0.47
Glutamine	4.28	3.41	3.47	4.59	3.94	4.33	3.39	0.81	0.93	0.78	0.39
Glycine	1.29	1.18	1.98	1.13	1.09	2.77	1.00	0.75	0.33	0.30	0.41
Hydroxyproline	0.32	0.16	0.45	0.47	0.41	0.48	0.25	0.16	0.16	0.56	0.59
Proline	0.72	0.59	0.71	0.79	0.78	1.20	1.09	0.26	0.16	0.99	0.86
Serine	0.14	0.15	016	0.09	0.11	0.25	0.07	0.06	0.58	0.35	0.29
Taurine	8.18	9.61	7.189	11.61	12.56	13.72	11.99	2.03	0.57	0.14	0.45
Tyrosine	1.02	0.67	1.17	1.21	0.99	1.50	1.04	0.29	0.16	0.44	0.66

1 Diet: methionine : cystine ratio relative to requirement (100)

2 All values are reported as LSMEANS as calculated by proc MIXED; MET = methionine intake, CYS = cystine intake, CYS(MET) = cystine intake within a methionine intake